EFFECTS OF OCEAN ACIDIFICATION ON DEVELOPMENT OF

ALASKAN CRAB LARVAE

By

Raphaelle Descoteaux

RECOMMENDED:

Dr. Jeremy Mathis

Dr. Sarah Hardy, Advisory Committee Co-Chair

Dr. Katrin Iken, Advisory Committee Co-Chair

Dr. Brenda Konar
Program Head, Marine Sciences and Limnology

APPROVED:

Dr. Michael Castellini
Dean, School of Fisheries and Ocean Sciences

Dr. John Eichelberger
Dean of the Graduate School

4/22/14
Date

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Raphaelle Descoteaux, B.S.

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Abstract

The oceans absorb a large proportion of the carbon dioxide gas (CO_2) emitted into the atmosphere. This CO_2 changes the chemistry of seawater to make it more acidic, a phenomenon termed ocean acidification. Ocean acidification can have negative impacts on marine fauna, especially during early life stages, presenting a risk to ecosystems and fisheries. This research tested the effects of ocean acidification on the larval development of three crab species in Alaska: Tanner crab (*Chionoecetes bairdi*), rock crab (*Glebocarcinus oregonensis*), and Dungeness crab (*Metacarcinus magister*). Experiments were undertaken to assess the effects of exposure to low-pH conditions (decrease of up to 0.6 pH units from current levels, range of pH ~8.1 to 7.5) on survival, growth (morphometrics and mass), and carapace mineral composition of larval Tanner, rock, and Dungeness crabs. Results showed a decrease in survival as well as a small but non-significant decrease in size of Tanner crabs. There was a small and complex effect of pH on survival of Dungeness crabs. Rock crabs raised in low-pH conditions (pH 7.5) had higher individual biomass than those raised in ambient conditions (pH 8.1). There was no significant impact of pH on mineralization of any species. Therefore, low pH had a negative effect on development of Tanner crabs, a small effect on Dungeness larval survival and no discernible negative effect on rock crab larvae. Differences in response to ocean acidification may be related to pre-adaptation to variable pH conditions through lifestyle such that species that live in deeper, more stable waters (e.g., Tanner crab) are more vulnerable than species living in shallower, more variable waters (e.g., rock and Dungeness crabs). These observations suggest that ocean acidification will have negative impacts on Tanner and Dungeness crab larval survival with potential implications for recruitment to the adult population and consequently, for their fisheries.

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Effects of ocean acidification on development of Alaskan crab larvae

Introduction

The increase in carbon dioxide (CO_2) concentration in the atmosphere caused by the burning of fossil fuel and deforestation is accompanied by a simultaneous increase in CO_2 concentration in the oceans (Feely et al., 2004). When this anthropogenic CO_2 reacts with seawater, it produces a weak acid (carbonic acid) that causes the pH of seawater to decrease. This phenomenon, termed ocean acidification (OA), is threatening marine ecosystems around the world (Fabry et al., 2008). The globally averaged pH of the ocean has already declined by 0.1 pH units since the beginning of the Industrial Revolution, and global models predict a decrease of another 0.3-0.4 pH units by the year 2100 (Caldeira and Wickett, 2003; Feely et al., 2009). This decline in seawater pH is accompanied by a reduction in the saturation state of calcium carbonate minerals, such as aragonite and calcite, which are essential for the calcification of shells and skeletal structures in many marine organisms. Although these chemical alterations to seawater occur globally, some regions are expected to be more affected than others. In particular, high-latitude waters will likely experience a greater decline in pH and calcium carbonate saturation states than tropical and subtropical waters (Fabry et al., 2009). Gas solubility increases with decreasing temperature so that cold, high-latitude waters absorb more CO_2 than in warmer regions. In addition, seasonally high primary productivity in high-latitude waters facilitates the draw-down of atmospheric CO_2 into seawater (Fabry et al., 2009; Mathis et al., 2011). Finally, large freshwater inflow from river and glacial sources further decreases the pH and calcium carbonate saturation states of seawater especially in coastal regions, exacerbating the OA effects (Mathis et al., 2011). However, the potential impacts of OA on marine organisms are largely unclear. Further understanding of the effects of OA is crucial to predicting future impacts to marine ecosystems and fisheries.

Populations of decapod crustaceans, including crabs, shrimps, and lobsters, sustain many major fisheries around the globe. Most crustaceans possess a mineralized carapace that

could make them vulnerable to decreases in carbonate ion concentrations in seawater. However, most crustacean species examined so far appear fairly tolerant to changes in pH (Whiteley, 2011). While calcification rate is expected to decrease with decreasing pH in most marine invertebrates, it actually increases in the blue crab *Callinectes sapidus*, the shrimp *Penaeus plebejus*, and the lobster *Homarus americanus* at CO_2 concentration of up to 2856 ppm (pH 7.31, Ries et al., 2009). The type of calcium carbonate mineral used and the magnesium/calcium ratio of the carapace are unaffected in these three species (Ries, 2011). Female red king crabs (*Paralithodes camtschaticus*) also exhibit positive effects of low pH (pH 7.7) on carapace mineralization, increasing in both calcium content and magnesium/calcium ratio (Long et al., 2013a). Additionally, hemolymph acid-base balance, osmotic regulation, oxygen consumption, growth, and carapace mineralogy of the burrowing shrimp *Upogebia deltaura* seem unaffected by CO_2 levels expected to occur by 2100 (pH 7.64, Donohue et al., 2012). In crustaceans, tolerance of calcification to reduced pH may be attributed to multiple characteristics, including the presence of a protective organic layer shielding the carapace from low-pH seawater, the ability to regulate pH in the exoskeletal compartment, and a high capacity for ion regulation (Ries et al., 2009; Whiteley, 2011). However, there are many exceptions to the apparent resistance of crustaceans to OA. Detrimental effects observed thus far in various crustaceans include decreased survival (Kurihara et al., 2008), decreased feeding rate (Appelhans et al., 2012), immune suppression (Hernroth et al., 2012), decreased heat tolerance (Metzger et al., 2007), alterations in behavior (de la Haye et al., 2011), and changes in gene expression (Fehsenfeld et al., 2011).

The diversity in responses among crustacean species suggests that effects are highly species-specific and may be partially attributed to differences in lifestyle (Whiteley, 2011). For example, the deep-water grooved Tanner crab (*Chionoecetes tanneri*) can only partially compensate for pH-induced hemolymph acidosis, while the shallow-water Dungeness crab (*Metacarcinus magister*) can fully compensate when exposed to the same degree of pH change (pH 7.1, Pane and Barry, 2007). Different responses to OA in

migratory versus non-migratory copepod species provide further evidence that organisms that are naturally exposed to a large range in pH are more tolerant to OA than those that only experience a narrow pH range in their natural environment (Lewis et al., 2013). Shallow water organisms that are naturally exposed to fluctuating pH might thus be able to tolerate OA better than species inhabiting deeper waters, which live in a comparatively more stable environment. However, intertidal organisms, while adapted to widely fluctuating conditions, may approach their limit of tolerance to environmental change (Hofmann and Todgham, 2010). To date, these alternative hypotheses remain mostly untested in decapod crustaceans with respect to pH.

Early life stages of marine invertebrates are often hypothesized to be more vulnerable to environmental changes than adults (Melzner et al., 2009; Ross et al., 2011). In decapod crustacean larvae, this vulnerability to low pH may be due to reduced capacity for osmo- and iono-regulation (Anger, 2001; Whiteley, 2011). Negative effects of reduced pH on crustacean larvae have been observed in a number of taxa. Juvenile red king and Tanner crabs (*Chionoecetes bairdi*) have reduced survival and growth with decreased pH (pH 7.5), although morphology remains unaffected (Long et al., 2013b). Juvenile Tanner crabs reared in low-pH conditions also contain less calcium than control crabs (Long et al., 2013b). Spider crab larvae (*Hyas araneus*) show reduced growth, fitness, and calcium incorporation, as well as delayed development, at low pH conditions (pH 7.35, Walther et al., 2010; 2011). The carapace of European lobster (*Homarus gammarus*) larvae is lighter and contains less calcium and magnesium when the larvae are raised under reduced pH conditions (pH 8.1, Arnold et al., 2009). While early life stages of many crustaceans grow slower and incorporate less calcium under low pH conditions, others respond positively. For example, larval size and calcium content increase with decreasing pH in red king crab larvae (Long et al., 2013a). The magnitude of these effects can even differ across latitudes and water temperatures for a single species. Spider crab larvae, for example, have a lower capacity for calcium incorporation under OA conditions at the northern end of their range than at lower latitudes (Walther et al., 2011). Despite the

growing number of studies of OA impacts on larval crustaceans, the effects of acidification on many commercially valuable crab species are still largely unknown.

Alaska is known for its crab fisheries and rich marine ecosystems. This study focuses on the early larval stage of three crab species found in Alaskan waters: Tanner crabs (*C. bairdi*), Dungeness crabs (*M. magister*), and rock crabs (*Glebocarcinus oregonensis*). Tanner crab is a large, commercially harvested species found in the Gulf of Alaska and Bering Sea and is closely related to the snow crab (*Chionoecetes opilio*), which also supports a valuable commercial fishery in Alaska. Dungeness crabs are also harvested in Alaska, in addition to sustaining fisheries in British Columbia, Washington, Oregon, and California. Rock crabs are too small to bear any economic importance, but are a predominant member of the rocky intertidal and shallow subtidal habitat along the west coast of North America. All three crab species play key ecological roles in their respective habitats, consuming a wide range of prey including mollusks (Yamada et al., 1993), crustaceans, and fish (Jewett and Feder, 1983; Stevens et al., 1982), and are themselves prey for other crustaceans (including cannibalism by older crabs, Jewett and Feder, 1983), octopus (Dodge and Scheel, 1999), fish (e.g., Livingston, 1989), and marine mammals (e.g., Garshelis et al., 1986). Crab larval stages can also prey on zooplankton (Paul et al., 1979) and other invertebrate larvae (Reed, 1969; Rumrill et al., 1985) as well as autotrophic and heterotrophic protists (Burnett and Sulkin, 2007; Sulkin et al., 1998), and are themselves consumed by fish (Morgan, 1990).

Life histories of all three crab species are fairly similar, differing mainly in the number, timing, and duration of developmental stages. After mating, females brood their eggs for several months (rock crabs, Knudsen, 1964) or up to about a year or more (Tanner and Dungeness crabs, Shirley et al., 1987; Swiney, 2008). Larvae of all three species are planktotrophic and pass through a series of zoeal stages (two zoeal stages for Tanner crabs (Haynes, 1981), five zoeal stages for Dungeness and rock crabs (Poole, 1966; Puls, 2001)), and all have one megalopa stage before transitioning to a benthic lifestyle. In the

benthos, juvenile crabs molt multiple times over the course of several months to years before reaching sexual maturity (Hines, 1991; Orensanz and Gallucci, 1988). Dungeness and rock crabs typically inhabit intertidal and shallow subtidal waters and are phylogenetically closely related (Family Cancridae), whereas Tanner crabs inhabit deeper waters and are more distantly related (Family Oregoniidae) (Azuma et al., 2011; Driskell, 1977; Harrison and Crespi, 1999). The different habitats of these three species range from intertidal to deep water, representing a gradient in the degree of natural, short-term pH fluctuations. Thus, comparing the effects of OA on these species may add to our understanding of the effects of exposure to natural pH variability on vulnerability to OA.

In this study, I examined whether the changes in seawater pH expected to occur in Alaskan waters during the next 100-200 years (expected decrease of 0.6 pH units, Caldeira and Wickett, 2003) are likely to impact the larval development of Tanner, Dungeness, and rock crabs. To address this question, I exposed newly-hatched crab larvae to experimentally-manipulated pH conditions and measured their survival, growth (as defined by morphometrics and mass), and mineral composition during the first zoeal stage. I hypothesized that survival, growth, and carapace mineralization in zoeae (from here on referred to simply as "larvae") of Tanner, Dungeness, and rock crabs would be negatively affected by OA, as observed in many other decapod crustacean larvae examined so far. Additionally, I hypothesized that impacts would be stronger in Tanner crabs, which live in deeper waters and are thus adapted to a more stable physical and chemical environment than the shallow-water species, which are naturally exposed to strong environmental fluctuations, including pH. To date, some research has assessed the impacts of low pH on Dungeness crabs and two members of the Tanner crab family (*C. bairdi* and *C. tanneri*) but these studies focused on juvenile and adult stages, not on larval stages (Long et al., 2013b; Pane and Barry, 2007). This study is the first to test the effects of OA on the larvae of Tanner, rock, and Dungeness crabs.

Methods

Study area

Experiments were conducted at the Kasitsna Bay Lab, a shared National Oceanographic and Atmospheric Administration (NOAA) and University of Alaska Fairbanks (UAF) facility located on the south shore of Kachemak Bay in south-central Alaska (Figure 1).

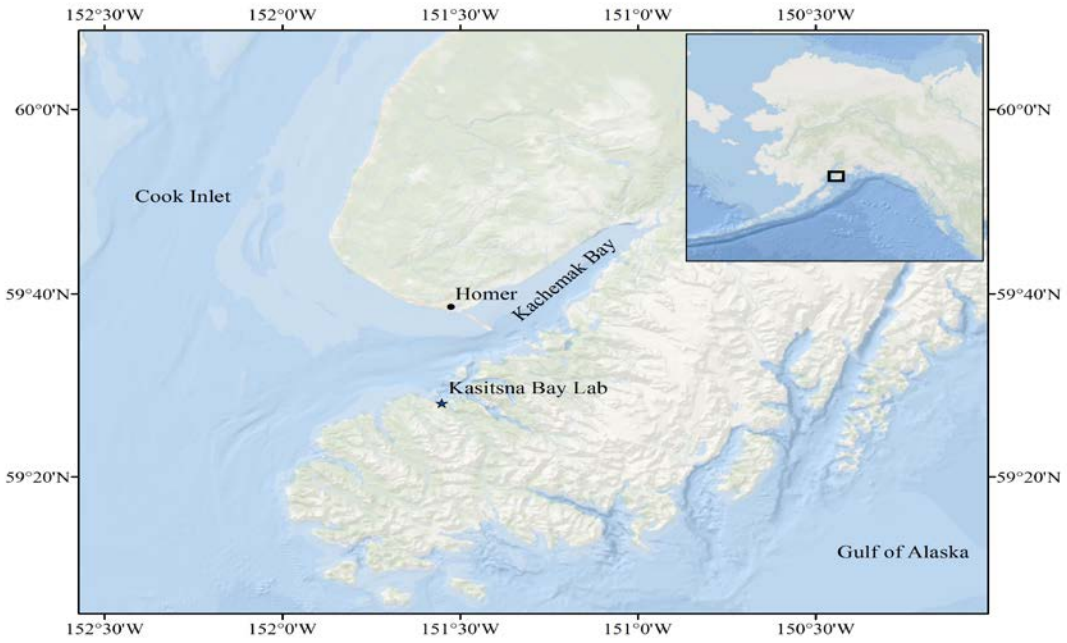


Figure 1: Map of study location. Gravid female crabs were collected in Kachemak Bay, Alaska and larval acidification experiments were undertaken at the Kasitsna Bay Lab depicted by the blue star on the map.

Kachemak Bay is a large glacial estuary subjected to one of the world's largest amplitude tidal ranges of up to 8 m, which can influence the physical characteristics to which shallow water organisms are exposed. Oceanic water enters the bay along the south shore and flows counterclockwise around the bay to eventually exit along its north shore (Burbank, 1977). High salinity and hard substrate characterize the south shore, while high

freshwater inputs lowering salinity and a sandy substrate characterize the north side (Spurkland and Iken, 2011). Areas of the central bay reach depths of up to 200 m. In nearby Prince William Sound, pH in spring 2009 ranged from ~8.05 in surface waters to ~7.95 at 200 m depth, with greater seasonality found in upper layers (Shake, 2011).

Although the bay and adjacent waters in Cook Inlet once supported a commercial fishery of Tanner and Dungeness crabs, severe stock declines forced closures of the commercial fisheries in 1994 and 1991, respectively. These fisheries have remained closed due to lack of stock recovery (Szarzi and Begich, 2004). A small sport fishery for Tanner crab reopened in 2008, but years of low abundance have again forced temporary closures.

Animal collection and larval culture

Larvae of all three species were obtained from ovigerous (egg-bearing) female crabs collected in Kachemak Bay, which were maintained in the lab until larvae were released. One female Tanner crab was collected on 3 May 2011 by the Alaska Department of Fish and Game using a sled dredge towed at 117 m depth. Twenty-three rock and one Dungeness female crabs were hand-collected at low tide in the intertidal zone on 17 May and 2 July 2011, respectively. The Tanner and Dungeness females were kept in individual aquaria with continuous flow of ambient seawater until eggs hatched. Because of their large numbers and smaller size, rock crabs were kept in individual plastic containers filled with seawater and placed in a large flow-through seawater bath to maintain ambient temperature. Water was changed daily in each container to maintain sufficient oxygen concentration and remove waste products. Female Tanner and rock crabs were given rocks for habitat and the Dungeness female was provided with soft sediment in which to burrow. All female crabs were fed to satiation with mussel and clam pieces supplied once per day.

In Alaskan waters, crab larvae hatch sequentially: Tanner crab larvae are released in late spring (April -May), followed by rock crab larvae (June -August), and then Dungeness

larvae (July -August) (Murphy and Iken, 2014; Stevens, 2003). Therefore, separate consecutive acidification experiments were conducted for each species. Experiments ran from 11-25 May 2011 for Tanner crab, 15 June to 2 July 2011 for rock crab, and 5-19 July 2011 for Dungeness crab. Larval hatching spanned 2-3 days for each female, but was not synchronous among the 23 rock crab females. For this species, larvae that all hatched on the same day were collected from four females and pooled such that larvae of the same age were used for the acidification experiment (see Table 1 for female information).

Table 1: Collection of broodstock. Location and time of female crab collection, female size expressed as carapace width and time of larval collection.

Common name	Scientific name	No	Site of collection	Date of female collection	Carapace width (mm)	Date of larval collection
Tanner crab	*Chionoecetes bairdi*	1	Kachemak Bay Trench	3 May 2011	109	11 May 2011
Rock crab	*Glebocarcinus oregonensis*	4	Little Tutka Bay	17 May 2011	21, 21, 23, 27	15 June 2011
Dungeness crab	*Metacarcinus magister*	1	MacDonald Spit	2 July 2011	103	5 July 2011

Once larval release was observed inside the plastic containers or aquaria, water flow was stopped, and water was replaced every 12 h. When a sudden increase in larval numbers indicated a peak of hatching, larvae were collected for the experiment. For each species, a total of 900 larvae were collected during peak hatching and carefully pipetted into seawater tanks for the acidification experiments (described below).

Only one Tanner and one Dungeness crab were available to produce larvae for the experiment, while the larvae of four females could be used for the rock crab experiment. In crustaceans, there is evidence that effects of OA can vary among broods of different females (Carter et al., 2013; Ceballos-Osuna et al., 2013; Pansch et al., 2013). Thus, larvae from a single female may not reflect the full range of species-specific responses to the pH treatments. However, running the experiments with the larvae from a single

female controlled for potential confounding effects from genetic variation among parents and/or differences in maternal care, which could have introduced a larger range of offspring phenotypes (Marshall et al., 2008). In this study, the number of females was dictated by availability, but future research should strive to test the effects of OA on larvae from multiple females to determine how representative these results are for the respective species.

Flow-through tank acidification experiments

Reduced-pH seawater was generated by bubbling CO_2 gas into natural seawater contained in a large header tank (Figure 2). The CO_2 flow into solution was regulated by a pH computer and solenoid valve (Aqua Medic). This reduced-pH water was used as the "low-pH" treatment (pH ~7.4 -7.6; approximately 0.6 pH units below ambient) in the experiments described below. A second header tank was filled with "ambient pH" seawater (pH ~8.0 to 8.2). Ambient pH was similar to pH found in nearby Prince William Sound in 2009 (pH 8.0 - 8.1, Shake, 2011). Water from the ambient and low-pH header tanks was gravity-fed into nine mixing tanks (Figure 2). In three of these nine tanks, ambient and low-pH waters were mixed to produce an "intermediate pH" treatment (pH ~7.8 to 7.9; approximately 0.3 pH units below ambient). Three mixing tanks contained ambient and three contained low pH water. For all treatments, the mixing tanks also allowed for stabilization of pH before water was fed into the experimental tanks. Water in every header and mixing tank was continuously mixed using aquarium pumps. Each mixing tank fed one smaller experimental tank (n=9, Figure 2). All seawater used in these experiments was filtered (Aqua-Pure AP110 filter) at 5-10 µm.

Three experimental tanks were randomly allocated to each of the three pH treatments: ambient, intermediate, and low pH. For the Tanner crab experiment, ten cylindrical PVC cores (~ 20 mL volume) with 333-µm Nitex mesh bottoms were suspended at the surface of each experimental tank as larval containers. For the rock and Dungeness crab experiments, larval containers were changed to 100-mL cups with 333-µm Nitex mesh

Figure 2: Experimental setup used in acidification experiments. One ambient and one low-pH header tank provided seawater to nine mixing tanks, which in turn fed nine experimental tanks where crab larvae were housed. Only five of the mixing and experimental tanks are visible in this photo.

bottoms to avoid crowding of the larvae. Water from the mixing tanks was dripped into each cup to ensure constant flow of pH-regulated water into the larval containers. A pH meter (VWR SympHony SB70P or SB80PD) outfitted with an Ag/AgCl pH glass electrode and an automatic temperature compensation (ATC) probe recorded the pH and temperature of each experimental tank every 30 min for the entire duration of the experiments (see Table 2 for a summary of pH and temperature data). The pH electrodes were calibrated approximately once per week. Temperature inside the tanks was not controlled, and thus fluctuated according to ambient conditions in Kachemak Bay (5.9-10.5 °C for the Tanner experiment, 8.1-12.8 °C for the rock crab experiment, and 9.3-11.0 °C for the Dungeness crab experiment). Oxygen saturation was checked regularly inside the tanks using a dissolved oxygen electrode (VWR SympHony).

Table 2: Summary of pH and temperature data. The Tanner crab experiment took place 11-25 May 2011, the rock crab experiment occurred 15 June to 2 July, 2011 and the Dungeness crab experiment occurred 5-19 July 2011. Data are presented as means ± one standard deviation. Temperature is given in °C.

		Tanner crab (n = 672)		Rock crab (n = 832)		Dungeness crab (n = 694)	
	Tank	pH ± SD	Temp ± SD	pH ± SD	Temp ± SD	pH ± SD	Temp ± SD
Ambient	1	8.14 ± 0.05	7.15 ± 0.33	8.02 ± 0.08	9.68 ± 0.88	8.09 ± 0.05	10.28 ± 0.45
	2	8.11 ± 0.05	7.28 ± 0.49	8.07 ± 0.05	9.50 ± 0.74	8.01 ± 0.03	10.08 ± 0.45
	3	8.20 ± 0.12	7.03 ± 0.65	8.01 ± 0.05	9.53 ± 0.80	8.05 ± 0.03	10.15 ± 0.48
Intermediate	1	7.95 ± 0.10	7.40 ± 0.70	7.80 ± 0.05	9.68 ± 0.70	7.75 ± 0.04	10.37 ± 0.49
	2	Not available	7.42 ± 0.76	7.79 ± 0.05	9.73 ± 0.67	7.76 ± 0.05	10.32 ± 0.49
	3	7.94 ± 0.12	7.20 ± 0.59	7.74 ± 0.06	9.55 ± 0.71	7.74 ± 0.03	10.11 ± 0.53
Low	1	7.64 ± 0.03	7.92 ± 0.68	7.56 ± 0.07	10.03 ± 1.12	7.40 ± 0.05	10.20 ± 0.59
	2	7.58 ± 0.08	7.76 ± 0.51	7.41 ± 0.08	9.92 ± 0.91	7.38 ± 0.05	10.60 ± 0.42
	3	7.65 ± 0.11	7.89 ± 0.71	7.51 ± 0.12	9.85 ± 0.89	7.64 ± 0.07	10.73 ± 0.63

For all experiments, ten crab larvae were placed in each holding cell for a total of 100 larvae per tank (ten cells per experimental tank) and 300 larvae per treatment (three tanks per treatment). Larvae were placed in the experimental tanks within 12 h of hatching and remained in the tanks either until they were sampled (see "Sampling and larval measurements" section below) or until they died. Larvae were fed approximately every two days with freshly-hatched *Artemia* nauplii at a density of approximately 1.0 nauplius mL^{-1}. Experiments were conducted in a laboratory with windows and, therefore, larvae were exposed to the natural photoperiod. Experiments lasted 15 days for Tanner crab, 18 days for rock crab, and 14 days for Dungeness crab larvae.

Closed-jar acidification experiments

In addition to the flow-through tanks described above, a second experiment for each species testing the same pH conditions was conducted simultaneously in 500-mL closed glass jars. This experiment was added to compare experimental setups as both flow-through and jars setups are commonly used in acidification experiments but it is unknown whether they yield similar results. Nine jars were used to replicate the flow-through design, and were filled with as little headspace as possible using water of the respective pH treatment obtained from the flow-through acidification system (n=3 jars per pH treatment). For each species, 900 larvae were collected within 12 h of hatching and divided equally among the nine jars (density: 100 larvae/500 mL). Jars were sealed and placed in a flowing seawater bath to maintain ambient temperature. For Tanner crab larvae, water pH was monitored every day using the pH electrode and meter described above, and water was replaced when pH started to drop below the desired levels, every 4-5 days. For the later rock and Dungeness crab experiments, however, it was determined that more frequent water changes would be preferable to reduce pH variability over time. Therefore, approximately half of the water from each jar was replaced with fresh seawater of the respective pH every day to maintain pH and oxygen levels and prevent buildup of waste products. Larvae were fed approximately every two days with freshly-hatched *Artemia* nauplii at about 1.5 nauplii mL^{-1}. Larvae from the jar experiment were

used for morphometric, mass and elemental composition measurements (described below), but survival rates were not recorded.

Sampling and larval measurements

Larvae were subsampled from both tank and jar experiments on days 0, 1, 2, 4, 6, 10, and 14 (or day 15 for Tanner crab larvae). For tank experiments, larvae were first counted inside the experimental cups to assess survival (Dungeness and rock crabs only, logistical constraints prevented regular survival counts for Tanner crab larvae), and then five live larvae from each tank (15 larvae per treatment) were removed using a large-mouth pipette. Larvae were transferred into a metal-free centrifuge tube partially filled with seawater of the experimental pH, and frozen for preservation. At the same sampling intervals, five larvae from each jar (15 larvae per treatment) were collected and preserved the same way. One centrifuge tube was used for all five larvae originating from each tank or jar. Frozen specimens were transported to the University of Alaska Fairbanks for morphometric, body mass, and mineral composition measurements (see below).

1. Survival

At days 0, 4, 6, 10, 14, and 18 for rock crabs and days 0, 1, 2, 4, 6, 10, and 14 for Dungeness crabs, surviving larvae were counted in each tank; survival was not measured for jar experiments. Logistical constraints prevented survival counts on other days in these experiments. Percent survival was calculated for each tank by dividing the number of surviving larvae by the number of larvae that should have been present given 0% mortality (adjusted for previously sampled larvae). Survival in Tanner crab flow-through experiments was only measured at the end of the experiment on day 15 by recording the number of larvae remaining in each tank as a fraction of the starting number.

2. Morphometric and body mass measurements

I measured and weighed larvae from each sampling event from flow-through tank and jar experiments for the sampling dates listed above. Frozen larvae were thawed and

photographed using a Leica DFC 420 digital camera (image size 2592x1944) attached to a Leica M165C dissecting microscope. Image J software was used to measure a series of body parts and appendages from the photographs: dorsal spine, rostral spine, lateral spine, antennae, body length, and abdominal segment length (Figure 3; measurements largely correspond to Webb et al., 2006). Only one of the two lateral spines and antennae were photographed for each larva. Larvae were then re-frozen for later body mass measurements.

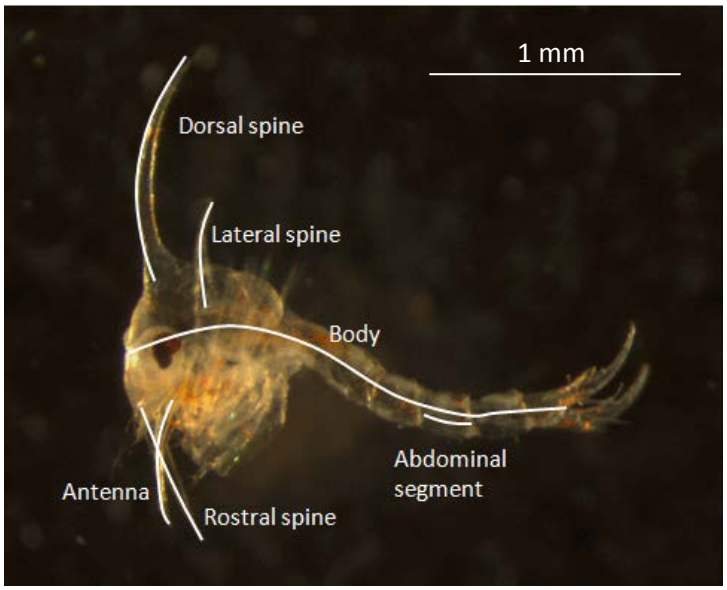


Figure 3: Larval morphometric measurements. Measurements taken for each crab larva included the lengths of the dorsal, rostral, and lateral spines as well as the lengths of the antenna, third abdominal segment, and the total body length. In addition to this lateral view, more photographs were taken to capture the lateral spine and antenna in a position perpendicular to the field of view.

Prior to weighing, each larva was thawed, rinsed a minimum of three times in ultra-purified water (Milli-Q) to remove salt residues, and dried at 40°C for a minimum of 24 h on a small pre-weighed glass slide (Electron Microscopy Science, 8 mm diameter, #1.5 thickness). The glass slide was then re-weighed with the larva on a Sartorius M2P

electronic microbalance (resolution 0.001 mg), and larval dry mass was determined by subtracting the mass of the slide.

3. Mineral composition

Mineral composition was measured for Tanner crab larvae (flow-through tanks on days 0, 2, 4, 6; jars on days 0, 2, 4, 6, 10), rock crab larvae (jars on days 0, 4, 6, 10), and Dungeness crab larvae (jars on days 0, 4, 6, 10). Analysis of all larvae was cost-prohibitive, so subsets of larvae were selected for measurements in order to make comparisons among crab species (all species from jar experiments) and between the two experimental setups (Tanner crab larvae raised in tanks vs. jars). Jars were used for inter-species comparisons so as to avoid using microalgae-fouled Dungeness crab larvae from the tank experiment.

Calcium and magnesium concentrations of larval carapaces were measured using a UP213 New Wave laser ablation system connected to an Agilent 7500ce inductively-coupled plasma mass spectrometer (ICP-MS) fitted with a cs lens stack. The laser was fired at a rate of 20 Hz with 80% output, travelling along the ablation path at a speed of 5 μm s^{-1}. A ~200 x 25-μm line was ablated along one fork of the telson at the tip of the abdomen (Figure 4). The laser gas stream was mixed with an internal standard (^{45}Sc, ^{72}Ge, and ^{89}Y) inside a spray chamber prior to entering the ICP-MS. Background counts of calcium and magnesium were measured during a 10-s pre-ablation period with zero laser power immediately prior to each analysis. Calcium and magnesium counts were averaged over the entire ablation period for each sample. Average background counts were subtracted from the average counts during ablation to obtain sample values. A FEBS-1 certified reference material (National Research Council Canada) was used to calculate calcium and magnesium concentrations from raw counts.

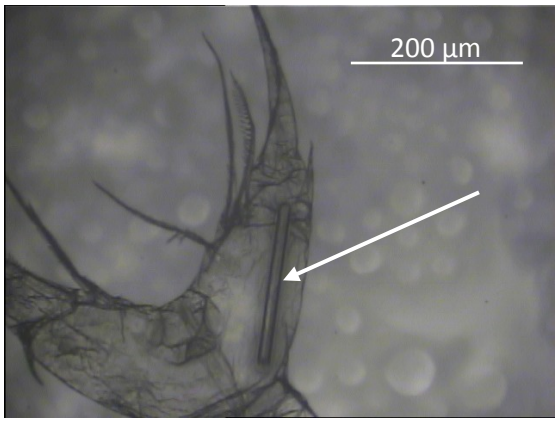


Figure 4: Laser ablation path for mineral composition analysis.

Statistical analysis

Differences in Dungeness and rock crab percent survival between pH treatments in the flow-through tanks were analyzed using two-way repeated measures ANOVAs with time as the repeated measure and pH as a fixed factor, using library lme4 in R (R Core Development Team, 2013). Time and pH were modeled as ordered factors. For body mass, morphometric measurements, and mineral composition measurements, two-factor repeated measures ANOVAs were performed with time as a repeated measure and pH as a fixed factor. Mineral (calcium and magnesium) data were log-transformed prior to analysis. Repeated measures ANOVAs for body mass, morphometric, and mineral composition measurements were performed using a mixed-effects model in R with the library nlme. For the Tanner crab flow-through tank experiment, the model was run without an interaction term because there were no low-pH larvae left to measure at day 15 and only one at day 10. Significance level was set at $\alpha = 0.05$ for all analyses, but the multiple ANOVA models for morphometric measurements required a Bonferroni adjustment of p-values to indicate this significance level for each experiment. Again, time and pH were modeled as ordered factors. In addition, PCA analyses were performed in Primer 6 by combining all length measurements to confirm overall effects of pH on crab morphometrics. All length measurements were normalized prior to analysis so that longer measurements such as body length would not dominate the analysis. PCA plots were

visually inspected for clustering of larvae according to pH. Finally, to assess whether morphometric, mass or mineral measurements changed through experimental time, regardless of pH, individual randomized block design ANOVAs with replicate tank as the blocking factor were run on larvae from ambient treatments only.

Results

Survival

Larval rock crabs survived for a maximum of 18 days and Dungeness crab larvae survived for a maximum of 14 days in flow-through tanks. On days 18 and 14, respectively, the last remaining larvae were sampled for body mass, morphometric, and mineral composition measurements. Survival did not differ significantly among pH treatments for rock crab larvae but there was a significant interaction between time and pH ($p = 0.0104$) in survival of Dungeness crab larvae (Figure 5). One of the low-pH tanks in the Dungeness crab experiment got heavily fouled with microalgae, which raised the pH to a value in between the intermediate and low-pH treatments (Table 2). When excluding this tank from the analysis, the pH signal became slightly stronger but overall results differed very little. Therefore, analyses presented here include this tank with relatively elevated pH. For Tanner crabs, although survival was not measured systematically throughout the experiment, only one larva remained in the low-pH treatment at day 10 and none remained at day 15. In contrast, 11 ambient and five intermediate-pH larvae survived until day 15. These results suggest that pH had a large impact on Tanner survival, a small and complex effect on Dungeness survival and no discernible effect on rock crab survival.

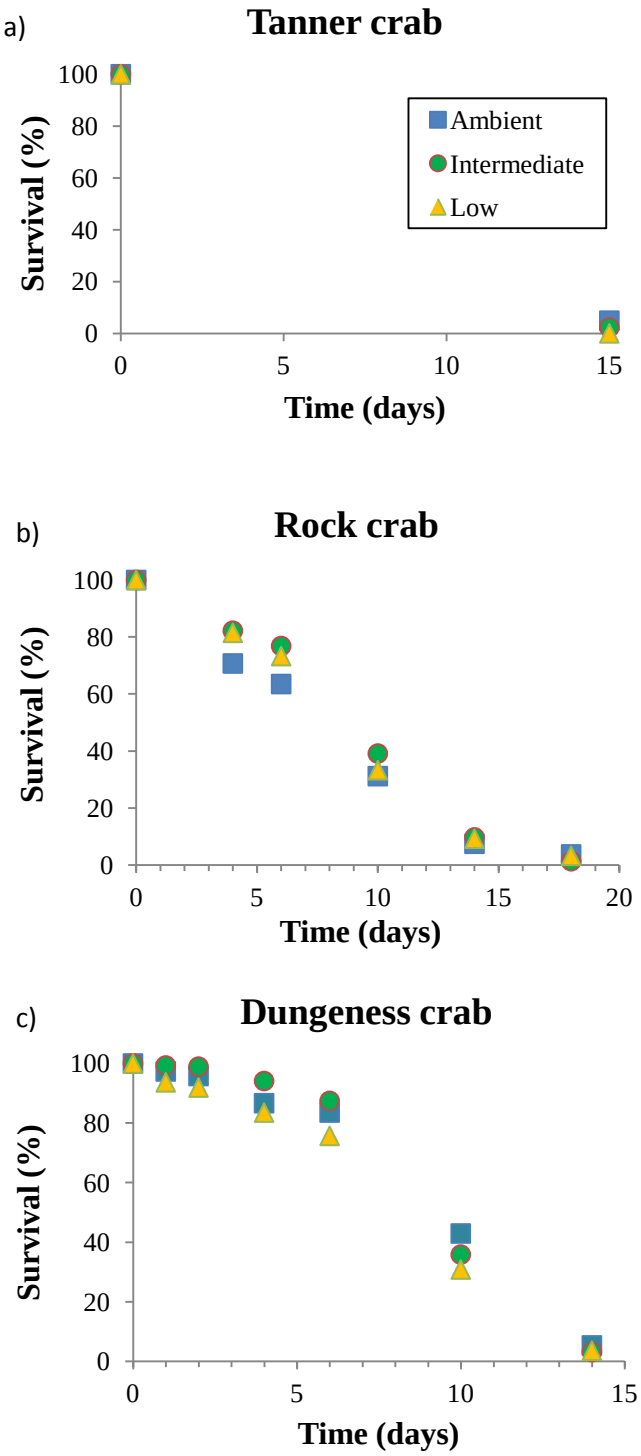


Figure 5: Survival of (a) Tanner, (b) rock, and (c) Dungeness crab larvae in tanks. Points are averages of survival across three replicate tanks for each pH treatment.

Growth

Tank experiment

The two-way repeated measures ANOVAs suggested that pH had little effect on larval size using various body measurements (Table 3). For Tanner crab larvae, however, the average lengths of the dorsal spine, rostral spine, and antenna were lowest in low-pH seawater (Figure 6, additional measurements are included as Figures A-1 to A-6 in appendix). This pH effect, however, was not significant at the Bonferroni-corrected $p = 0.0083$ and was small in magnitude (less than 5% difference between low-pH and ambient treatments averaged over the whole experimental period). PCA analysis confirmed a small but observable effect of pH on larval morphometrics in Tanner crab larvae (Figure 7a). Data from the low-pH treatment, while mostly overlapping with the ambient and intermediate treatments, were slightly separated from the other treatments along the PC1 axis, indicating some separation among antennae, rostral, lateral, and dorsal spine lengths. PCA plots for rock and Dungeness crabs showed no clustering by pH treatment (Figure 7b, c). pH did not significantly impact larval mass (Table 4, Figure 8). There were no significant interactions between pH and time on any measurements.

Randomized block design ANOVAs showed that most length measurements in Tanner, rock, and Dungeness crabs raised in ambient conditions did not change significantly over time except for dorsal ($p = 0.0180$) and lateral spine ($p = 0.0469$) lengths in Tanner crabs and body length ($p = 0.0039$) in Dungeness crabs. There was a significant increase in mass over the course of the experiments for Tanner ($p < 0.0001$) and rock crabs ($p = 0.0009$). These observations suggest that mass is plastic but that length measurements are for the most part set within a larval stage. However, no increase in mass was observed for Dungeness crabs, which were only weighed up to day 6 because of an unexpected algal bloom occurring in the tanks that fouled a large number of larvae and impeded accurate mass measurements.

Table 3: ANOVA table for morphometric measurements in the tank experiments. Highlighted p-values are significant at an overall $\alpha = 0.05$ (Bonferroni-adjusted $p < 0.0083$).

Tank experiments		Time			pH			Interaction		
		df	F-value	p-value	df	F-value	p-value	df	F-value	p-value
Tanner	Dorsal spine	32	4.10	0.0055	4	6.09	0.0611	No interaction term		
	Rostral spine	32	1.34	0.2744	4	10.07	0.0274			
	Lateral spine	32	2.33	0.0656	4	2.84	0.1710			
	Antenna	32	2.23	0.0758	4	5.14	0.0785			
	Abdominal segment	32	1.32	0.2794	4	1.06	0.4273			
	Body	32	2.83	0.0317	4	2.06	0.2423			
Rock	Dorsal spine	30	1.80	0.1421	4	0.22	0.8123	30	0.79	0.6366
	Rostral spine	30	1.00	0.4328	4	1.16	0.4013	30	1.38	0.2356
	Lateral spine	30	1.10	0.3809	4	0.06	0.9470	30	0.72	0.6970
	Antenna	30	2.24	0.0766	4	0.42	0.6810	30	0.85	0.5891
	Abdominal segment	30	2.30	0.0701	4	1.51	0.3250	30	0.80	0.6288
	Body	30	4.16	0.0055	4	1.19	0.3935	30	0.50	0.8753
Dungeness	Dorsal spine	28	2.40	0.0619	4	0.40	0.6966	28	1.03	0.4438
	Rostral spine	28	1.37	0.2647	4	0.18	0.8424	28	1.77	0.1140
	Lateral spine	28	1.07	0.3957	4	0.98	0.4492	28	1.39	0.2360
	Antenna	No antenna measurements								
	Abdominal segment	28	1.31	0.2865	4	1.41	0.3449	28	0.50	0.8750
	Body	28	0.53	0.7500	4	1.28	0.3725	28	1.46	0.2066

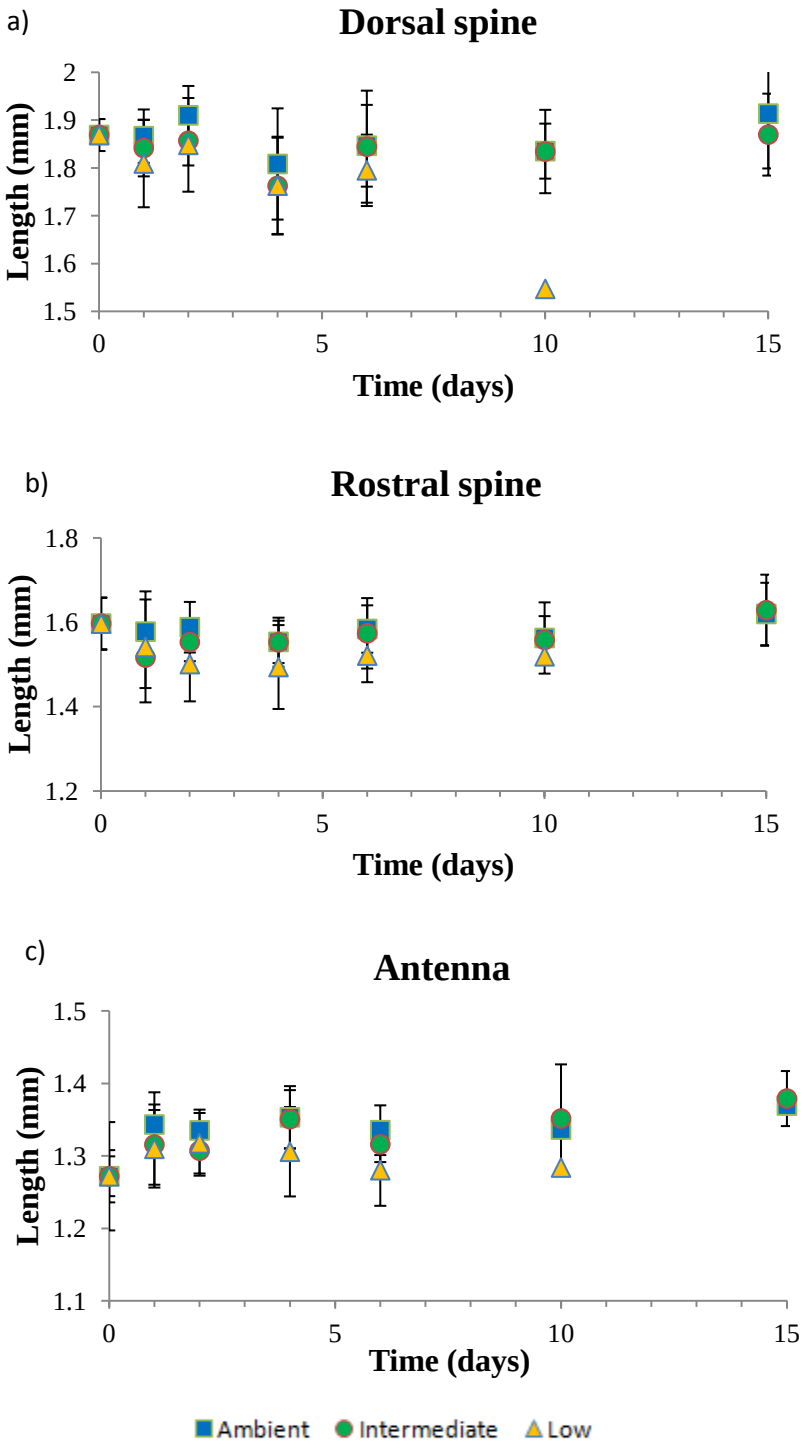
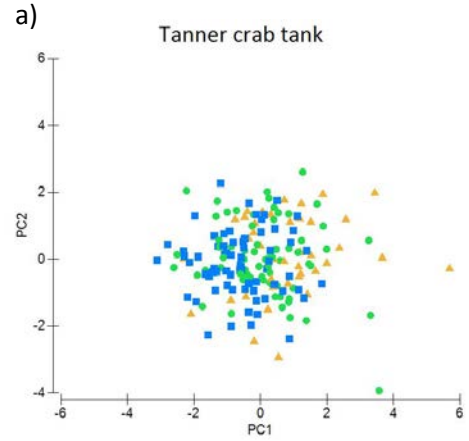


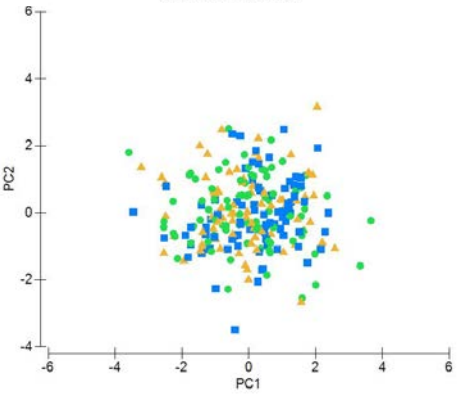
Figure 6: Length of the (a) dorsal spine, (b) rostral spine, and (c) antenna in Tanner crab larvae. Error bars are ± one standard deviation.

a)



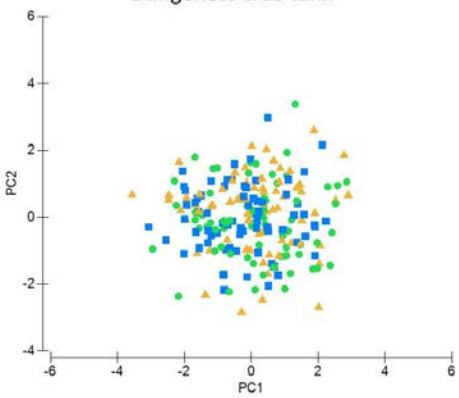
Variable	PC1	PC2
Dorsal	-0.435	-0.260
Rostral	-0.540	-0.109
Lateral	-0.511	-0.106
Segment	-0.116	0.737
Body	-0.211	0.604
Antenna	-0.448	0.029
Eigenvalues	1.79	1.18
%Variation	29.8	19.7
Cum.%Variation	29.8	49.5

b)



Variable	PC1	PC2
Dorsal	-0.461	-0.260
Rostral	-0.492	-0.017
Lateral	-0.501	0.041
Segment	-0.032	0.751
Body	-0.240	0.593
Antenna	-0.485	-0.122
Eigenvalues	1.61	1.13
%Variation	26.8	18.9
Cum.%Variation	26.8	45.7

c)



Variable	PC1	PC2
Dorsal	-0.355	-0.571
Rostral	-0.325	-0.445
Lateral	-0.459	-0.210
Segment	-0.449	0.580
Body	-0.596	0.309
Eigenvalues	1.67	1.21
%Variation	33.5	24.2
Cum.%Variation	33.5	57.7

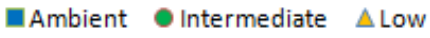


Figure 7: Principal component analysis (PCA) of size of crab larvae raised in tanks. Morphometric measurements used in analysis include lengths of the dorsal spine, rostral spine, lateral spine, antennae, abdominal segment, and body for (a) Tanner, (b) rock, and (c) Dungeness crab larvae.

Table 4: ANOVA table for body mass measurements in the tank experiments. Highlighted p-values are significant at $\alpha = 0.05$.

	Time			pH			Interaction		
	df	F-value	p-value	df	F-value	p-value	df	F-value	p-value
Tanner	31	6.43	0.0003	4	4.18	0.1047	No interaction term		
Rock	30	15.60	<0.0001	4	2.04	0.2449	30	0.62	0.7830
Dungeness	15	7.59	0.0026	4	0.16	0.8584	15	0.79	0.5883

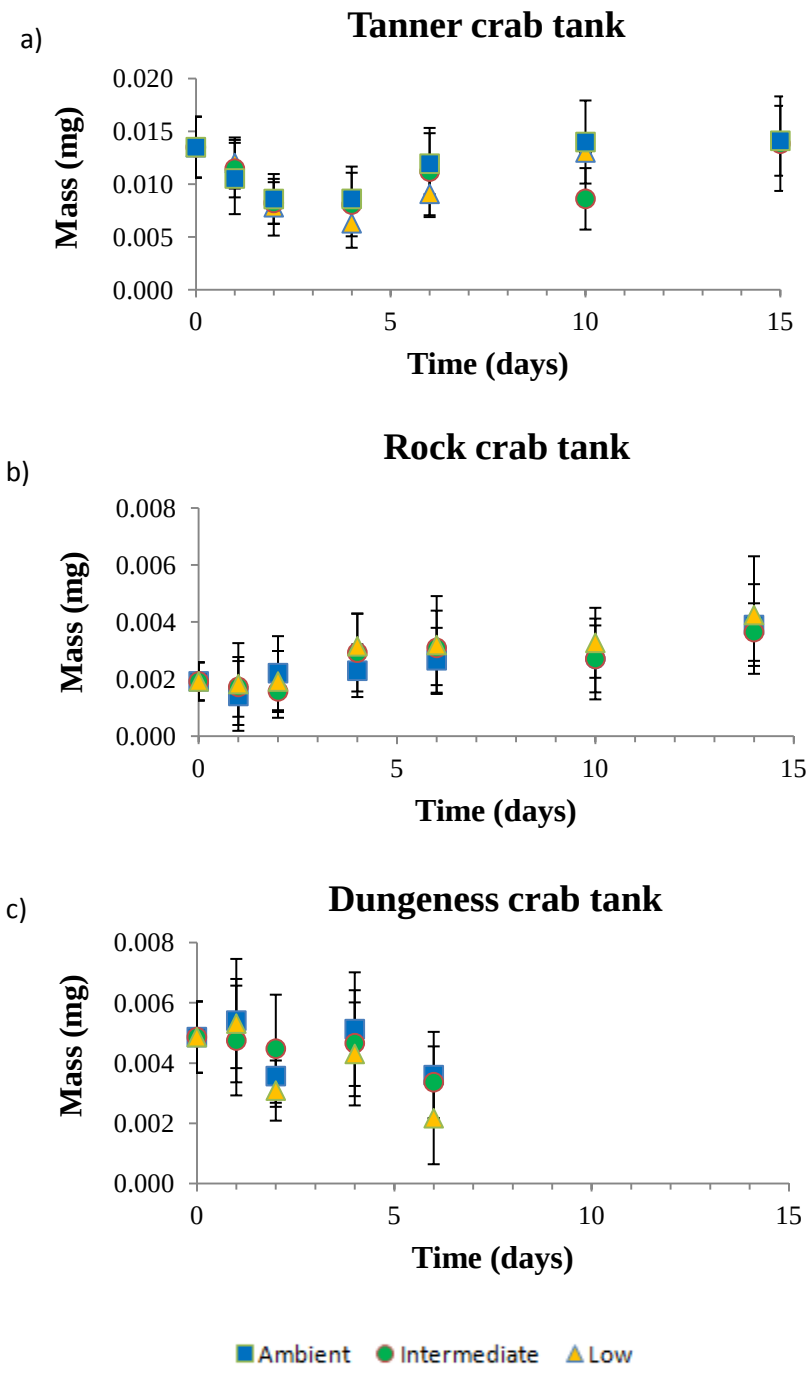


Figure 8: Mass of (a) Tanner, (b) rock, and (c) Dungeness crab larvae raised in tanks. Points are average values for each pH treatment and error bars are ± one standard deviation. Dungeness crab larvae raised in tanks were only weighed until day 6 because algal fouling prevented accurate mass measurements beyond that date.

Jar experiment

pH had no significant effect on any length or mass measurement in the jar experiments (Table 5, 6), except for rock crab mass ($p = 0.0456$). When averaged over the entire experimental period, rock crab mass was highest in the low-pH treatment and lowest in the intermediate pH treatment (Figure 9). There were no significant interactions between pH and time on any measurements. Examination of PCA plots confirmed the absence of separation in length measurements among pH levels (Figure 10). Together, these observations suggest that pH had no effect on morphometrics and mass in Tanner and Dungeness crabs raised in jars, while it had a positive effect on mass in rock crabs.

As in tank experiments, length measurements of larvae raised in ambient conditions in jars did not vary over time except for body ($p < 0.0001$) and antenna ($p = 0.0043$) lengths in Tanner and dorsal spine lengths in rock ($p < 0.0001$) and Dungeness crabs ($p = 0.0005$). Overall, mass increased over the course of the experiments for all species ($p < 0.0001$). Again, these observations suggest that body mass can increase within a larval stage (i.e., between molts), but length measurements remain for the most part constant.

Mineral content

Calcium and magnesium concentrations varied widely among species, experiments, and individuals, but pH did not significantly affect concentrations of either element in the larval carapace (Table 7, Figure 11-12). Under ambient conditions, calcium concentrations increased significantly over time for Tanner larvae in tanks ($p = 0.0007$) and jars ($p < 0.0001$), and in rock ($p = 0.0025$) and Dungeness ($p = 0.0191$) crabs in jars. Magnesium concentration increased significantly in Tanner larvae raised in tanks ($p = 0.0264$) and jars ($p < 0.0001$) as well as in Dungeness larvae raised in jars ($p = 0.0034$), and did not change significantly in rock crab larvae in jars. These results indicate that in almost all cases, calcium and magnesium concentrations can change within a larval stage and that the absence of pH response cannot be attributed to lack of plasticity. Calcium concentrations were always higher than magnesium concentrations, confirming that the

Table 5: ANOVA table for morphometric measurements in the jar experiments. Highlighted p-values are significant at an overall $\alpha = 0.05$ (Bonferroni-adjusted $p < 0.0083$).

Jar experiments		Time			pH			Interaction		
		df	F-value	p-value	df	F-value	p-value	df	F-value	p-value
Tanner	Dorsal spine	30	2.32	0.0677	4	0.16	0.8589	30	0.42	0.9253
	Rostral spine	30	5.37	0.0012	4	0.87	0.4842	30	1.54	0.1755
	Lateral spine	30	2.30	0.0693	4	0.44	0.6734	30	0.67	0.7460
	Antenna	30	2.10	0.0914	4	1.20	0.3848	30	1.20	0.3378
	Abdominal segment	30	0.90	0.4968	4	1.24	0.3811	30	0.84	0.5955
	Body	30	1.97	<0.0001	4	0.03	0.9751	30	1.97	0.0734
Rock	Dorsal spine	30	10.53	<0.0001	4	0.21	0.8193	30	1.34	0.2559
	Rostral spine	30	0.84	0.5313	4	0.64	0.5720	30	1.23	0.3116
	Lateral spine	30	4.51	0.0035	4	0.35	0.7218	30	1.32	0.2654
	Antenna	30	3.18	0.0203	4	0.22	0.8128	30	2.00	0.0693
	Abdominal segment	30	1.18	0.3420	4	0.52	0.6319	30	1.34	0.2571
	Body	30	1.04	0.4122	4	1.21	0.3876	30	0.31	0.9740
Dungeness	Dorsal spine	30	5.09	0.0017	4	3.88	0.1155	30	0.96	0.4957
	Rostral spine	30	2.54	0.0495	4	0.08	0.9236	30	0.24	0.9894
	Lateral spine	30	5.41	0.0012	4	0.50	0.6415	30	1.73	0.1190
	Antenna	30	1.23	0.3190	4	0.25	0.7893	30	0.96	0.4959
	Abdominal segment	30	3.66	0.0106	4	0.63	0.5791	30	0.50	0.8772
	Body	30	9.44	<0.0001	4	0.28	0.7725	30	0.79	0.6364

Table 6: ANOVA table for body mass measurements in the jar experiments. Highlighted p-values are significant at $\alpha = 0.05$.

	Time			pH			Interaction		
	df	F-value	p-value	df	F-value	p-value	df	F-value	p-value
Tanner	30	54.58	<0.0001	4	1.13	0.4094	30	1.83	0.0985
Rock	28	8.62	<0.0001	4	7.37	0.0456	28	1.17	0.3516
Dungeness	30	13.31	<0.0001	4	2.34	0.2120	30	0.86	0.5745

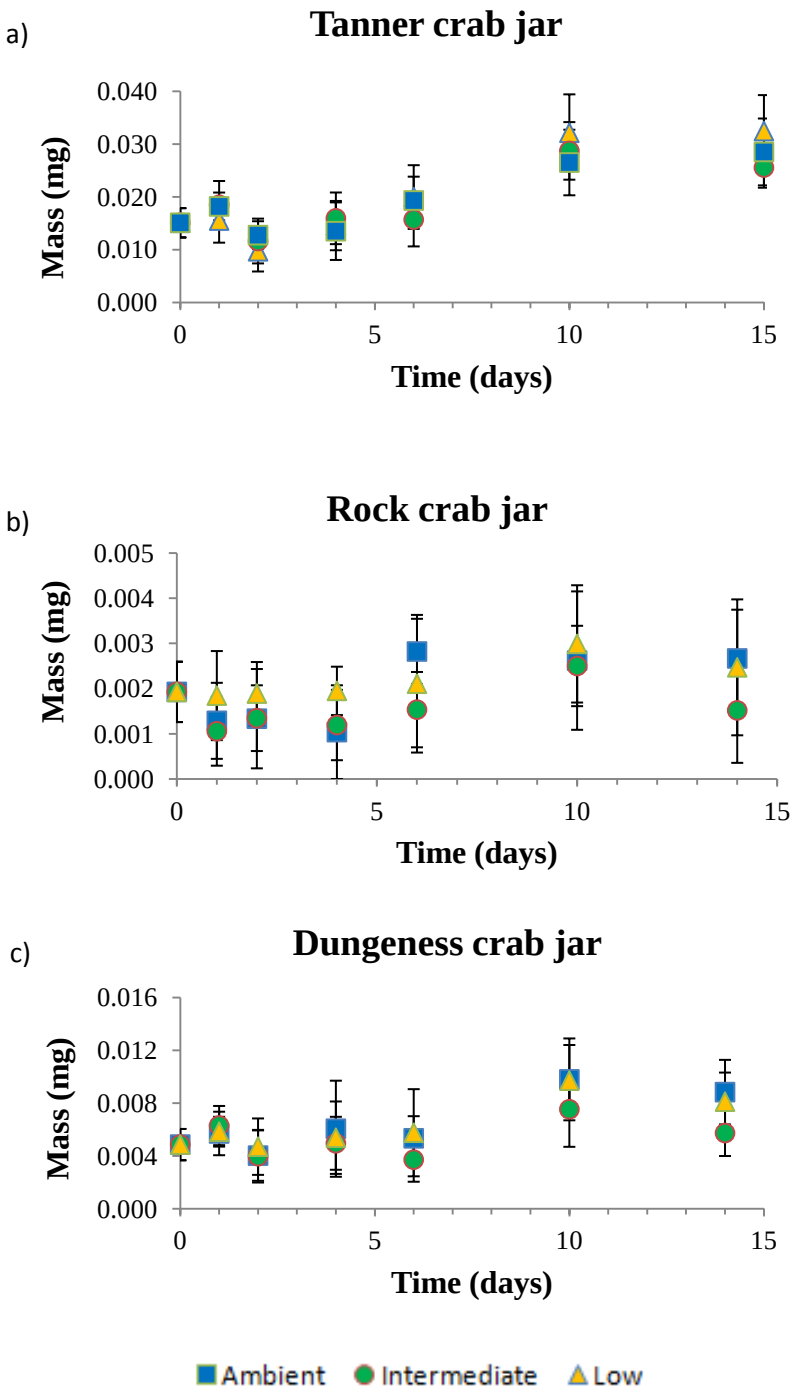
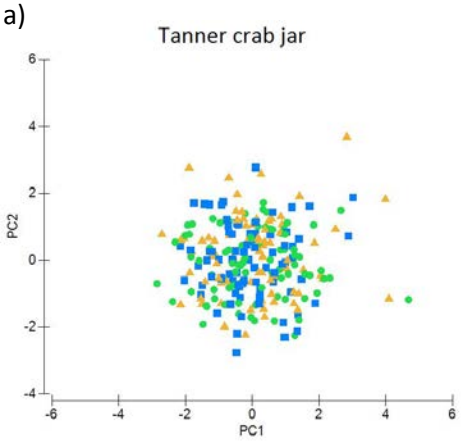


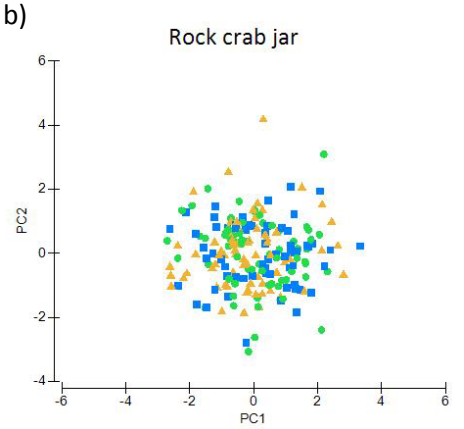
Figure 9: Mass of (a) Tanner, (b) rock, and (c) Dungeness crab larvae raised in jars. Points are average values for each pH treatment and error bars are ± one standard deviation.

a)



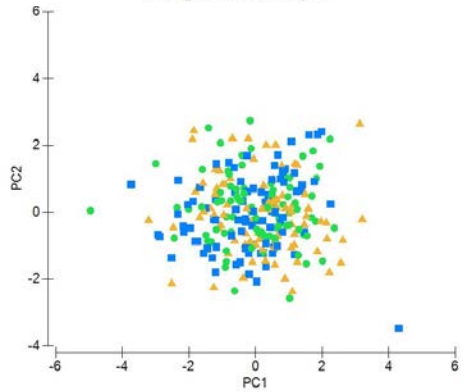
Variable	PC1	PC2
Dorsal	-0.459	0.325
Rostral	-0.549	0.062
Lateral	-0.282	-0.061
Segment	-0.223	-0.626
Body	-0.231	-0.665
Antenna	-0.553	0.230
Eigenvalues	1.46	1.17
%Variation	24.3	19.4
Cum.%Variation	24.3	43.8

b)



Variable	PC1	PC2
Dorsal	-0.499	-0.397
Rostral	-0.349	-0.350
Lateral	-0.394	0.292
Segment	-0.237	0.469
Body	-0.329	0.614
Antenna	-0.556	-0.195
Eigenvalues	1.49	1.09
%Variation	24.9	18.2
Cum.%Variation	24.9	43.1

c)



Variable	PC1	PC2
Dorsal	0.484	0.142
Rostral	0.488	-0.200
Lateral	0.375	-0.388
Segment	0.359	0.379
Body	0.345	0.638
Antenna	0.373	-0.488
Eigenvalues	1.7	1.19
%Variation	28.3	19.8
Cum.%Variation	28.3	48.2

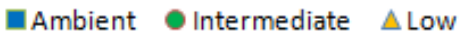


Figure 10: Principal component analysis (PCA) of size of crab larvae raised in jars. Morphometric measurements used in analysis include lengths of dorsal spine, rostral spine, lateral spine, antennae, abdominal segment, and body for (a) Tanner, (b) rock, and (c) Dungeness crab larvae.

Table 7: ANOVA table for calcium and magnesium concentrations. Measurements were taken for Tanner (tank and jar experiments), rock (jar experiment) and Dungeness (jar experiment) crab larvae. Highlighted p-values are significant at $\alpha = 0.05$.

Calcium		Time			pH			Interaction		
Species	Experiment	df	F-value	p-value	df	F-value	p-value	df	F-value	p-value
Tanner	Tanks	11	2.39	0.1378	4	0.04	0.9659	11	0.11	0.9782
	Jars	18	21.38	<0.0001	4	1.86	0.2687	18	0.92	0.5022
Rock	Jars	12	8.88	0.0043	4	2.29	0.2172	12	1.37	0.3002
Dungeness	Jars	12	13.56	0.0008	4	1.17	0.3986	12	0.58	0.6809

Magnesium		Time			pH			Interaction		
	Experiment	df	F-value	p-value	df	F-value	p-value	df	F-value	p-value
Tanner	Tanks	11	9.77	0.0036	4	0.74	0.5338	11	1.73	0.2129
	Jars	18	24.93	<0.0001	4	3.19	0.1486	18	1.32	0.2971
Rock	Jars	12	1.13	0.3545	4	1.57	0.3130	12	2.32	0.1163
Dungeness	Jars	12	16.9	0.0003	4	2.11	0.2362	12	0.57	0.6887

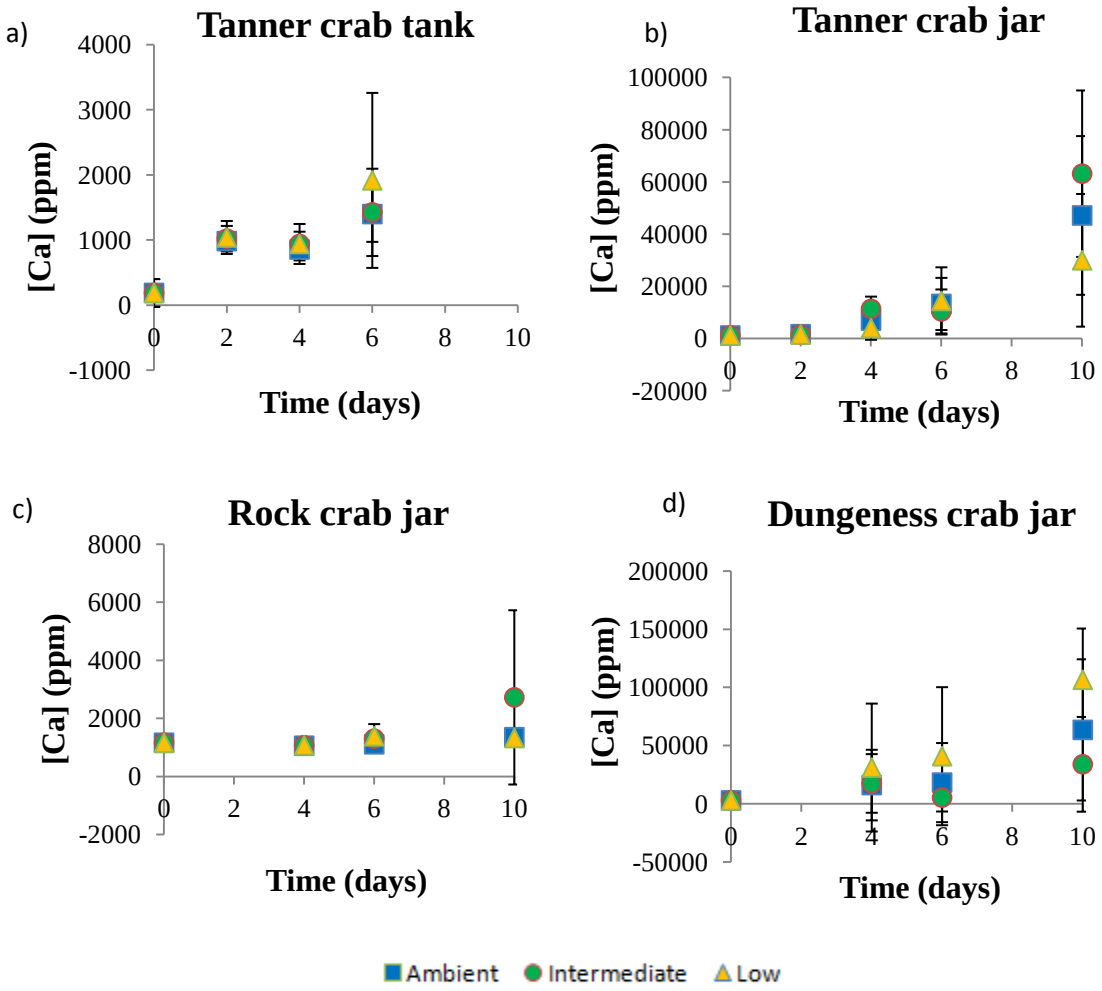


Figure 11: Calcium concentrations in larvae. Measurements were made for Tanner larvae raised in (a) tanks and (b) jars as well as in (c) rock crab and (d) Dungeness crab larvae raised in jars. Error bars are ± one standard deviation.

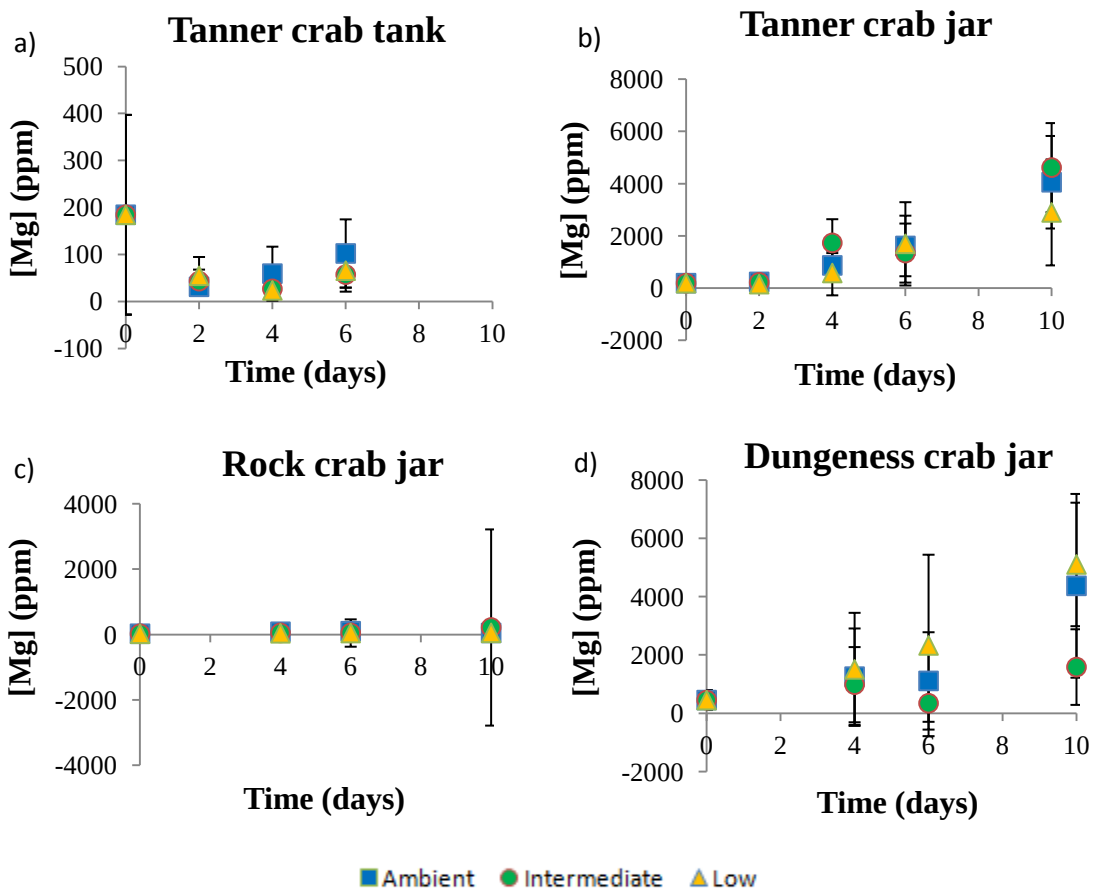


Figure 12: Magnesium concentrations in larvae. Measurements were made for Tanner crab larvae raised in (a) tanks and (b) jars as well as in (c) rock crab and (d) Dungeness crab larvae raised in jars. Error bars are ± one standard deviation.

readings were not dominated by sea salt that had not been removed from the carapace during washing. In addition, calcium and magnesium values were much higher in Tanner crab larvae from the jar experiment compared to the tank experiment, indicating that the tank setup may have stressed larvae more than the jar setup. At days 2, 4, and 6, respectively, there were about 2, 8, and 8 times more calcium and 5, 29, and 21 times more magnesium in Tanner larvae raised in jars compared to tanks.

Examination under a stereomicroscope revealed variable appearance of the dried larval exoskeleton within Tanner and Dungeness crabs. While some larvae were transparent and crumpled (Figure 13a), others looked shiny and inflated (Figure 13b). For Tanner and Dungeness jar experiments, the incidence of the “shiny” larvae increased over time from 0% at day 0 to > 63% at day 10. For Tanner crabs at day 10, there were fewer “shiny” larvae in the low-pH treatment (33% of larvae) than in the ambient (78%) and intermediate (89%) treatments. However, the opposite pattern was observed in Dungeness larvae at day 10, when 89% of low-pH larvae were “shiny” while only 56% and 44% were “shiny” in ambient and intermediate pH treatments, respectively. The “shiny” larvae were never observed in rock crabs and only one such Tanner larva was found in tank experiments. This change in appearance over time may represent different stages in the molting cycle within the zoea I stage, such that the “crumpled” larvae are in the post-molt stage after hatching whereas the “shiny” larvae are in the intermolt stage (Anger, 2001). This latter group yielded, on average, higher calcium and magnesium readings with mean calcium and magnesium being about 11 and 4 times higher, respectively, in “shiny” than in “crumpled” Dungeness crab larvae at day 10. In Tanner crabs, calcium and magnesium were about 3 and 2 times higher, respectively, in “shiny” than in “crumpled” larvae.

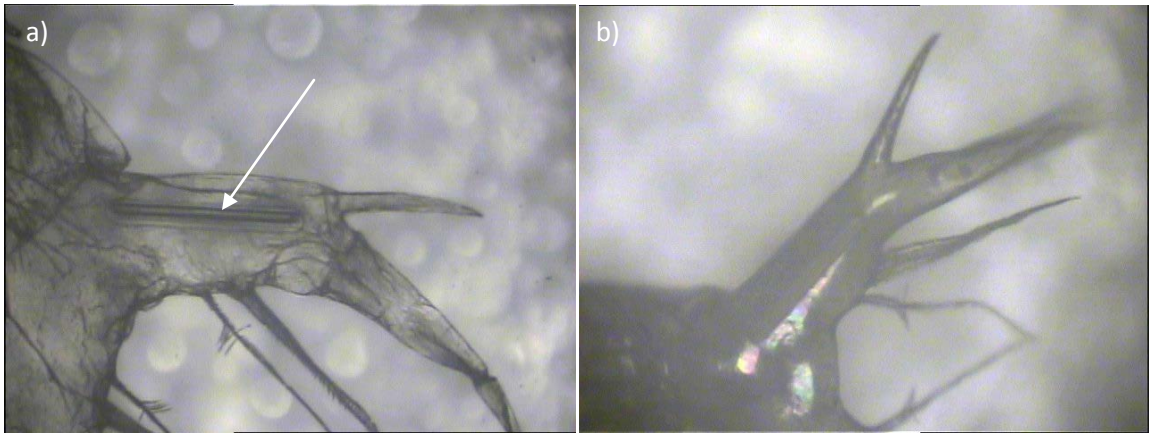


Figure 13: Appearance of the larval exoskeleton. Tip of the larval abdomen of (a) a translucent and crumpled-looking larva and (b) a shiny and inflated-looking larva. The white arrow points to the laser ablation line used for elemental analysis.

Discussion

In this study, pH affected survival of Tanner and, to a lesser extent, Dungeness crab larvae, but not rock crabs. In addition, pH had a small negative effect on morphometrics of Tanner crab larvae, and positively affected rock crab larval mass. OA thus has the potential to affect the survival and growth of crab larvae, depending on species, but has no apparent effect on mineralization within the first zoeal stage in any of the investigated species. Results partially support the first hypothesis that crab larvae are vulnerable to OA, and support the second hypothesis that Tanner crabs are the most vulnerable of all three species tested. The observed differences in response to pH support the notion that the effects of OA vary among decapod crustacean species.

Experimental considerations

The purpose of a controlled experiment is to keep all conditions constant except for the variable under investigation, but an experimental setup can introduce stressors that affect the viability of the study organisms. Regardless of effects of pH, survival was very low for all treatments, especially in the tank experiments, indicating that additional stressors

may have been present. Crab larvae often have high mortality in the field or in the laboratory (e.g., Shirley and Shirley, 1989), but the mortality rates observed here were higher than expected given higher survival rates in previous studies (e.g., Sulkin and McKeen, 1989). Natural mortality can be caused by a variety of factors such as predation (Morgan, 1990), diet (Anger et al., 1981), temperature and salinity (e.g., Reed, 1969), and pathogens (e.g., Talpur et al., 2011).

While predators were excluded in this experiment, larvae could have suffered from cannibalism. Cannibalism is a well-known phenomenon in larvae of some crab species (Epelbaum and Borisov, 2006), but is more common during later stages of development (e.g., Zmora et al., 2005). Larval numbers decreased over time in experimental tanks (even accounting for subsampling), but no dead larvae were observed, suggesting they may have been consumed. The incidence of cannibalism could be related to diet. In this experiment, larvae were fed freshly hatched *Artemia* nauplii, which are commonly used as a crab larval food source in laboratory experiments (Anger et al., 1981; Epelbaum and Borisov, 2006) but are not a natural food source in the wild. In the laboratory, Dungeness crab larvae can survive and molt successfully on a diet of *Artemia* nauplii (Sulkin et al., 1998). Nonetheless, *Artemia* nauplii may be difficult to ingest, or may not satisfy nutritional requirements. Larval density can also affect survival during laboratory rearing. However, in *Carcinus maenas*, a stocking density of 94 larvae L^{-1}, which is comparable to densities in the present study, achieved a 75% survival to the megalopa stage (Galley et al., 2011). Temperatures found in the experimental system were within a range that favors survival, at least for Dungeness crabs, so are unlikely to have caused high mortality (Reed, 1969). Salinity was not controlled or monitored during these experiments, but is not expected to have deviated substantially from the range commonly experienced in the wild. Pathogens could also have reduced survival as no antibiotics were used. In addition, turbulence in conjunction with the mesh screen inside each experimental cup could have stressed the larvae and reduced survival in the flow-through system.

Responses of larval crabs to pH differed markedly between flow-through tank and jar experiments. Negative effects of pH on Tanner crab morphometrics were only observed in the flow-through tank experiment, while a positive effect on mass in the low-pH treatment was only observed in jars, indicating that the flow-through tanks could have been a more stressful environment than the jars. In addition, calcium and magnesium concentrations differed widely between Tanner crabs raised in tanks and those raised in jars, suggesting that differing conditions in the two experiments produced these differing results. The most notable difference between experimental set-ups was that turbulence was much greater in the flow-through tanks than in the closed jars. Turbulence may have impacted larval survival by limiting ability for prey capture and by physically damaging larvae. While these observations suggest that a jar setup may be preferable for larval studies, the advantage of a flow-through system is the constantly replenished pH-adjusted water, which reduces pH or oxygen level fluctuations due to metabolism. Results from this study suggest that the experimental setup can have a large impact on the outcome of an OA study and investigators should carefully consider additional stressors caused by the setup, such as turbulence in a flow-through system. Further, these results indicate that negative effects of OA on Tanner crab larvae may only become apparent when pH changes act in conjunction with additional stressors. Ocean acidification in nature is expected to occur concurrently with a range of other environmental changes in marine waters, including changes in temperature, salinity, ocean mixing, and food availability, and effects of combined stressors frequently act synergistically to reduce an organism's fitness (Harley et al., 2006; Zittier et al., 2013).

Effects of OA on larval crabs

Tanner, rock, and Dungeness crab larvae in this study responded differently to low pH, suggesting that effects of OA are species-specific. Species specificity was mostly detectable from differences in survival and growth responses as there was no significant impact of pH on mineral composition. Rock crab larval survival was not affected by pH, and similar resilience has also been observed in larvae of the northern shrimp, *Pandalus*

borealis (Arnberg et al., 2013; Bechmann et al., 2011), the porcelain crab, *Petrolisthes cinctipes* (Ceballos-Osuna et al., 2013), the spider crab, *H. araneus* (Schiffer et al., 2013), and the European lobster, *H. gammarus* (Arnold et al., 2009). In contrast, pH reduced survival in Tanner and, to a lesser extent, Dungeness crab larvae. OA exposure during embryonic and larval development similarly reduces survival of red king crab larvae, *P. camtschaticus* (Long et al., 2013a). In addition, some species exhibit no effects of OA at the larval stage, but have reduced survival in later stages. In porcelain crabs, for example, larval survival is not affected, but juvenile survival decreases by 30% after exposure to low pH conditions (pH 7.6, Ceballos-Osuna et al., 2013). Lower pH could thus further reduce survival at later stages of development in the crab species investigated here.

Reduced survival in crab larvae could be caused by a change in metabolic costs associated with OA. Shallow-water crabs typically possess efficient iono-regulation machinery, enabling them to deal with variable environmental conditions and high levels of physical exertion (Whiteley, 2011). This machinery may also help prevent large deviations in extracellular pH due to OA (Whiteley, 2011). Differences in survival among Tanner, rock, and Dungeness crabs could have been caused by different capacities for iono-regulation. Nonetheless, iono-regulation could entail large energetic costs during long-term exposure to OA, which may lead to decreased survival if not compensated by increased feeding.

OA also appears to affect growth in a species-specific manner. Dungeness larvae showed no effect of pH on morphometrics and mass, yet there were small effects on Tanner crab larval morphometrics and rock crab larval mass. Tanner crab larvae reached a smaller size in the low pH treatment, whereas rock crab larvae were slightly heavier under low pH conditions. Some species show no mass or length response to reduced pH (e.g., porcelain crab larvae (pH 7.6, Carter et al., 2013; Ceballos-Osuna et al., 2013), northern shrimp larvae (pH 7.6, Arnberg et al., 2013)), while others show reduced growth (e.g., American lobster (Keppel et al., 2012), European lobster (Arnold et al., 2009)), and still

others show increased size (e.g., red king crab (Long et al., 2013a)). Reduced mass gain is sometimes caused by a reduction in mineral (calcium and magnesium) incorporation into the exoskeleton (Arnold et al., 2009). However, none of the three species studied here showed changes in mineral incorporation with pH, so the slight increase in mass at low pH in rock crabs can probably be explained by an increase in soft tissue mass rather than in the exoskeletal mineral component.

Larval length measurements obtained here were consistent with other studies (Shirley et al., 1987; Webb et al., 2006), but body mass measurements did not match those of previous studies. Dry mass of laboratory-raised and wild-caught Tanner crab zoea I stage are typically on the order of 50 to 60 µg (Incze and Paul, 1983; Incze et al., 1984), whereas their average dry mass in this study was only ~10 µg. No literature values were found for Dungeness or rock crab larval mass. Measurements presented here were taken after freezing and thawing larvae, likely affecting larval body mass through cell rupture and soft tissue loss. However, this loss was likely consistent among pH treatments and sampling times, so the observed treatment effects remain valid.

The reduced length of the rostral spine, dorsal spine, and antennae observed in Tanner crabs could have important repercussions on larval survival and fitness in the wild, especially if effects became more severe in later stages. Larval spines can deter predators by increasing the effective size of the larvae so that they are more difficult to engulf (Bullard et al., 1999; Morgan, 1989). Shorter spines could make the larvae more susceptible to predators with repercussions on survival and recruitment to the adult population.

Reduced pH caused only very small, non-significant changes in morphometric measurements in Tanner crab larvae, and no discernible changes in the other two species. Decapod larvae increase in size most prominently at molting, so the failure to detect a size effect could be the result of larvae not surviving to molt to the second larval stage. I

did not observe molting during the course of my study, which is not surprising given that the length of the experiments was shorter than the average time to molting to zoea II stage, at least for Tanner and Dungeness crabs (Lovrich and Ouellet, 1994; Poole, 1966). Therefore, large changes in lengths of spines and other body parts would have been unlikely in the two-week experimental period. In this study, only a few length measurements changed significantly over time in the ambient treatment, which further supports the idea that there is little plasticity in body size within one larval stage and that effects of pH on larval size would likely be most noticeable after molting. Indeed, in spider crab and European lobster larvae, the effects of OA on growth only become measurable in later stages of larval development (Arnold et al., 2009; Walther et al., 2010). Carry-over effects have also been observed where exposure to low pH during one life stage has repercussion on survival in following stages (Long et al., 2013a). Because all larvae died before their first molt in this study, I could not observe any long-term effect of pH on the larvae. In contrast to length measurements, body mass increased over time in the ambient treatments (except for Dungeness larvae raised in tanks), confirming previous observations that some mass increase occurs between larval molts (Anger, 2001; Incze et al., 1984; Lovrich and Ouellet, 1994). This observation suggests that body mass is not fixed within a molting cycle and that environmental stress such as OA could alter mass during the first zoeal stage. Therefore, the lack of a discernible mass change with pH is likely real and not an artifact of a discrete growth pattern.

Contrary to survival and growth, the response of mineral composition to OA did not differ among the three species tested. Shell calcification can be one of the most sensitive processes to OA. Low pH reduces various measures of calcification in many marine invertebrate taxa, including mollusks (e.g., Gazeau et al., 2007), echinoderms (e.g., Courtney et al., 2013), corals (Chan and Connolly, 2013), and crustaceans (e.g., Arnold et al., 2009). However, I detected no effect of pH on calcium or magnesium concentrations in the larval carapace, consistent with the idea that crustaceans are generally well protected from OA impacts on exoskeleton mineralization (Whiteley, 2011).

Decapod crustacean larvae typically begin calcification at the megalopal stage (at least in species for which early life stages are planktonic), possibly because heavy calcification could weigh down the larvae and impair buoyancy (Anger, 2001). Nonetheless, larvae may still incorporate a small amount of minerals into their exoskeleton, even at the zoeal stages (Anger, 1984). Calcium and magnesium found here in larval Tanner, rock, and Dungeness crabs may represent the beginnings of shell calcification; however, the possibility that these minerals were actually contained in other structures, such as the underlying soft tissues or the carapace chitin-protein matrix, cannot be ruled out. If these minerals were in fact part of the early calcification process, then it appears that OA does not affect calcification in early larval development of Tanner, rock, and Dungeness crabs. These results contrast with studies on European lobster larvae, whose partially calcified carapace contains less calcium and magnesium in low pH conditions (Arnold et al., 2009). Spider crab larvae also incorporate less calcium when exposed to low pH conditions, but effects vary depending on temperature, larval stage, and among populations (Walther et al., 2011). In contrast, larval red king crabs incorporate more calcium under low pH conditions (Long et al., 2013a). This response is similar to that of several adult decapod crustaceans for which calcification increases with decreasing pH (Long et al., 2013a; Ries et al., 2009). While pH did not affect calcium content in larval Tanner crabs in this study, it decreased calcification in juvenile Tanner crabs (Long et al., 2013b), indicating that different developmental stages of the same species can react differently to OA.

Inter-specific and ontogenetic variability in response to OA

Mounting evidence suggests that vulnerability to environmental change can be more acute in species that live in stable environments (Hofmann and Todgham, 2010). Organisms that are naturally exposed to fluctuating pH conditions may possess compensatory mechanisms that could enable them to deal with OA. A varying capacity for acid-base regulation explains at least some of the inter-specific variability observed in

adult crabs (Whiteley, 2011). Indeed, when exposed to low pH seawater, a shallow-water crab species can recover from hemolymph acidosis much better than a deep-sea crab species (Pane and Barry, 2007). Similarly, copepod species that undertake extensive vertical migrations through water layers of differing pH are more tolerant to OA than non-migratory species that experience a more consistent pH (Lewis et al., 2013).

Results from this study support the idea that lifestyle and/or habitat influence resistance to effects of OA. Tanner crabs showed the greatest degree of vulnerability to OA with reduced survival and slightly reduced size at low pH, whereas Dungeness crabs only showed a temporary reduction in survival, and rock crabs showed no negative impacts of low pH at all. In Kachemak Bay, Tanner crabs are typically found deeper than Dungeness crabs (Richard Gustafson, personal communication; Driskell, 1977). I collected all three species used in this study in the same estuary but I found the Tanner crab female at greater depth (> 100 m) than rock and Dungeness crab females, which were both collected in the intertidal zone. Crabs in nearshore waters can experience extensive pH variability due to freshwater runoff, metabolic processes (photosynthesis and respiration), and upwelling of deep low-pH waters (Feely et al., 2010; Mathis et al., 2011; Shake, 2011). Changes in pH can be even greater and occur faster in very shallow waters. Tidepool pH, for instance, can drop dramatically (~ 0.6 pH units change) within the course of just a few hours (Congleton, 1980). Therefore, of the species examined here, those that are naturally exposed to a greater range in pH in the wild were also more resilient to OA. Data presented here support the hypothesis that adaptation to a stable environment predisposes a species to OA sensitivity. However, this adaptation may be most applicable to adults as the larval habitat is similar among all three species (upper water column, Murphy and Iken, 2014) and could thus not explain differences in OA responses. It is currently unclear whether this adaptation actually spans all life stages. An alternative hypothesis suggests that, while intertidal organisms may be well adapted to a large range of environmental conditions, including pH, they may also be more vulnerable to change because they are already maximizing their ability to deal with extreme

conditions (Hofmann and Todgham, 2010). My results do not support this alternative hypothesis.

Many marine invertebrates undergo ontogenetic shifts in habitat use, which may help explain differences in responses to OA among life stages of the same species. In vertically migrating copepods, for example, life stages that migrate extensively (adults) show more tolerance to OA than non-migratory stages (nauplii) (Lewis et al., 2013). Early life stages could also be more vulnerable to OA because of their reduced capacity for osmo-regulation. In the mangrove crab, *Sesarma curacaoense* the capacity for osmo-regulation increases gradually throughout development (Anger and Charmantier, 2000). If this ontogenetic pattern is consistent across crab species, and tolerance to pH variations is related to the capacity for iono- and osmo-regulation, then it is likely that early life stages of other crabs will also be more susceptible to OA. However, even early life stages can be relatively well equipped to deal with hypercapnia in many tolerant taxa (Melzner et al., 2009). Additionally, in porcelain crabs, habitat change through ontogeny does not correlate well with sensitivity to OA, suggesting that more factors must be at play (Ceballos-Osuna et al., 2013).

In conclusion, Tanner and Dungeness crab larvae experienced reduced survival, and Tanner crab larvae also grew less when exposed to the lower pH conditions expected to occur in the next 100-200 years in Alaskan waters. Rock crab larvae appeared to be more resistant to these conditions. OA, therefore, has the potential to affect larval development of some crab species but may leave others relatively unaffected. These results emphasize the specificity of species responses and highlight the need for future studies to identify patterns in species vulnerability to OA. In addition, these results show potential negative impacts of OA on larval development of commercially valuable crab species. Reduced larval size and survival could lead to reduced recruitment into the adult population, which could in turn negatively impact crab fisheries. Therefore, OA has the potential to alter

marine ecosystems and may directly impact commercially and ecologically important crab species.

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Appendix

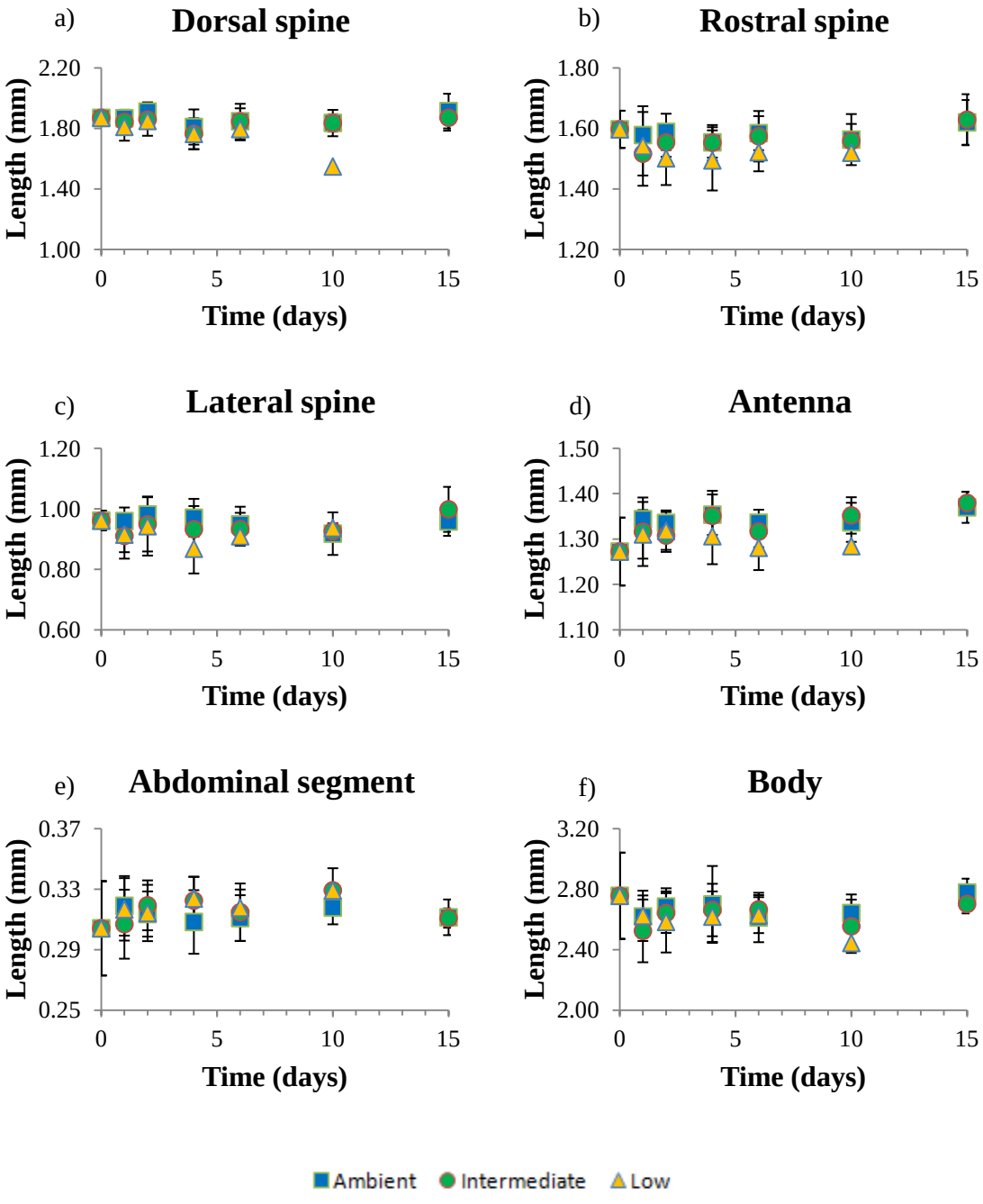

Figure A-1: Morphometric measurements of Tanner crab zoeae raised in tanks. Measurements include (a) dorsal spine length, (b) rostral spine length, (c) lateral spine length, (d) antenna length, (e) length of the abdominal segment and (f) total body length. Error bars represent ± one standard deviation.

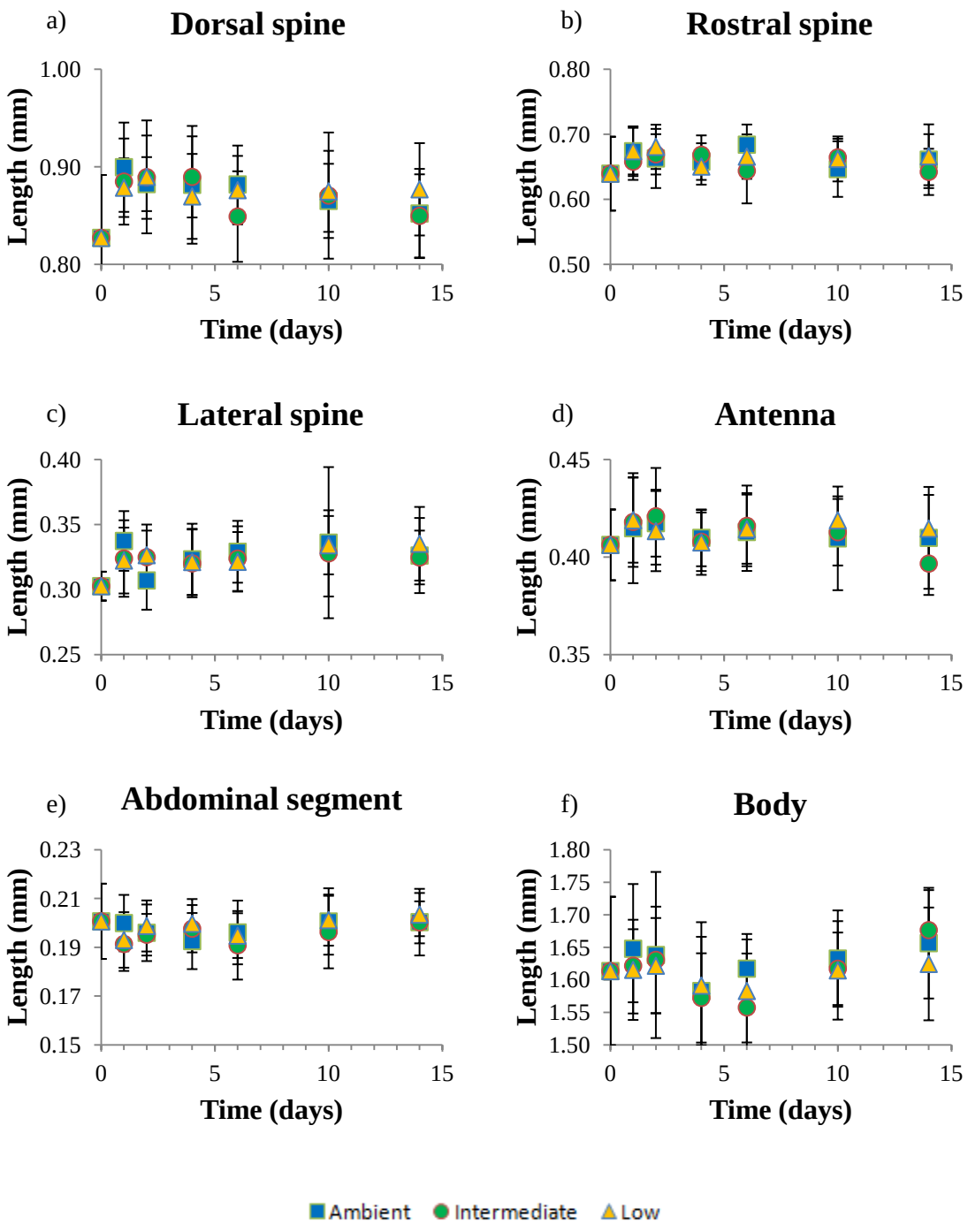

Figure A-2: Morphometric measurements of rock crab zoeae raised in tanks. Measurements include (a) dorsal spine length, (b) rostral spine length, (c) lateral spine length, (d) antenna length, (e) length of the abdominal segment and (f) total body length. Error bars represent ± one standard deviation.

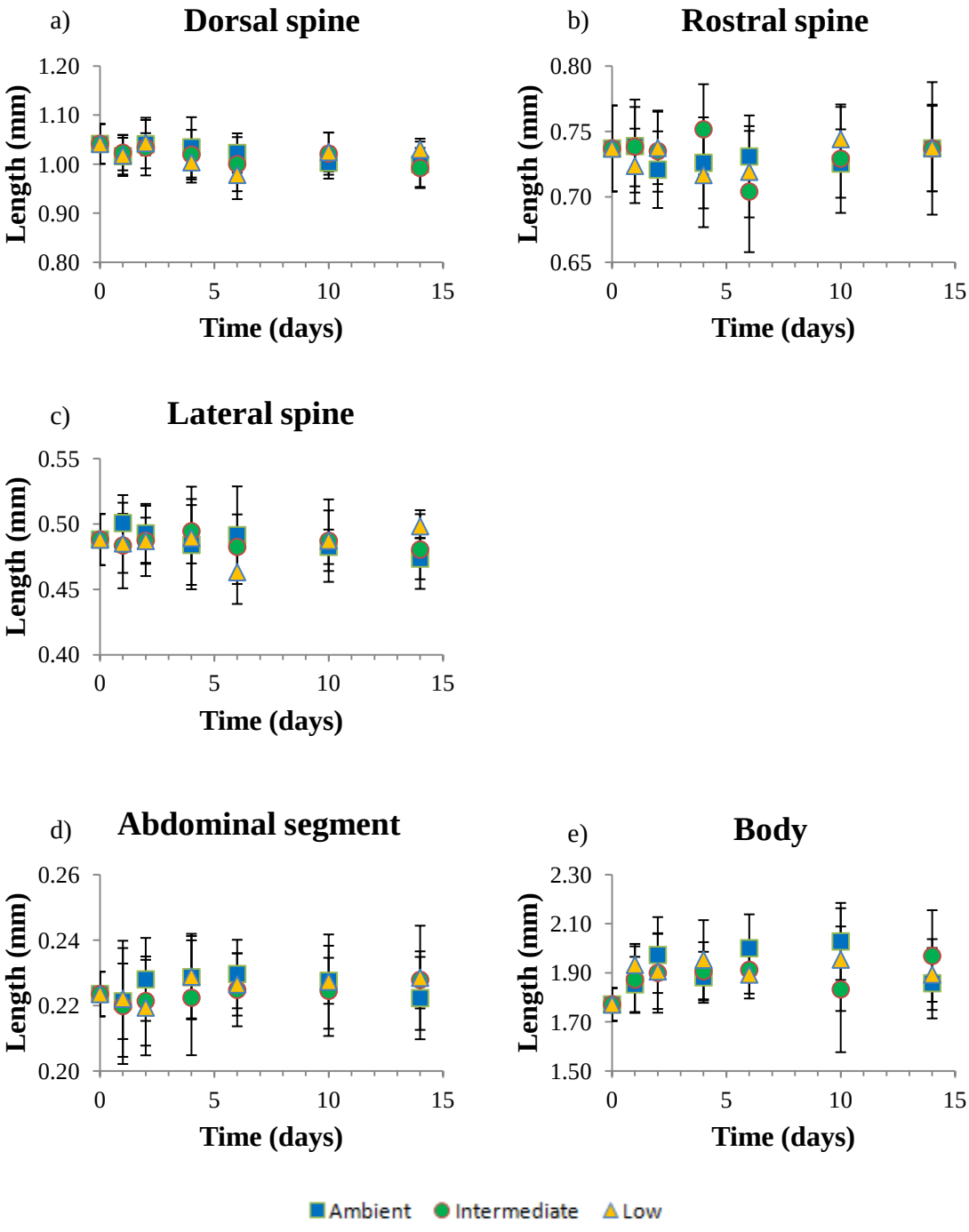

Figure A-3: Morphometric measurements of Dungeness crab zoeae raised in tanks. Measurements include (a) dorsal spine length, (b) rostral spine length, (c) lateral spine length, (d) length of the abdominal segment and (e) total body length. Error bars represent ± one standard deviation. Antenna length was not measured for Dungeness zoeae raised in tanks.

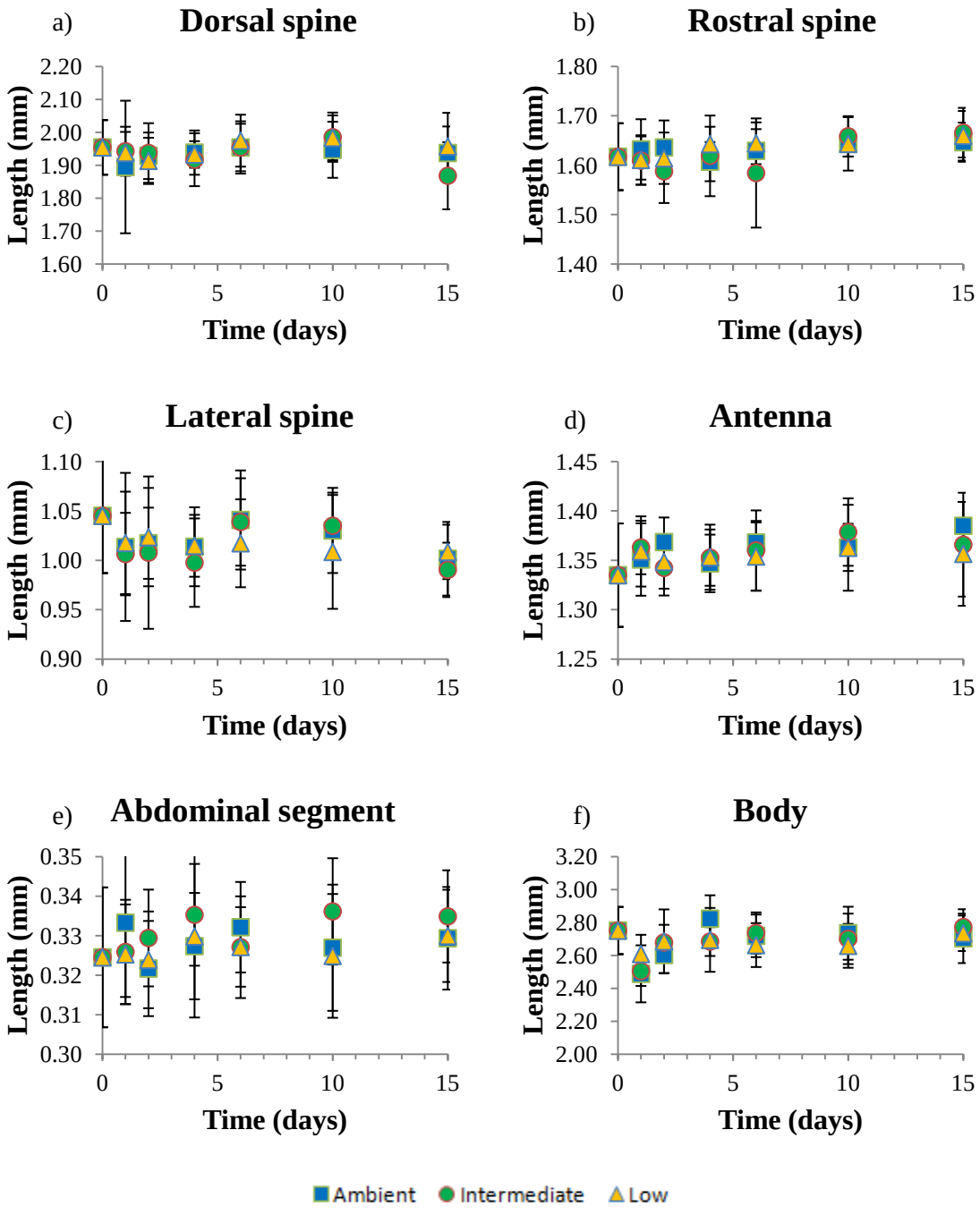

Figure A-4: Morphometric measurements of Tanner crab zoeae raised in jars. Measurements include (a) dorsal spine length, (b) rostral spine length, (c) lateral spine length, (d) antenna length, (e) length of the abdominal segment and (f) total body length. Error bars represent ± one standard deviation.

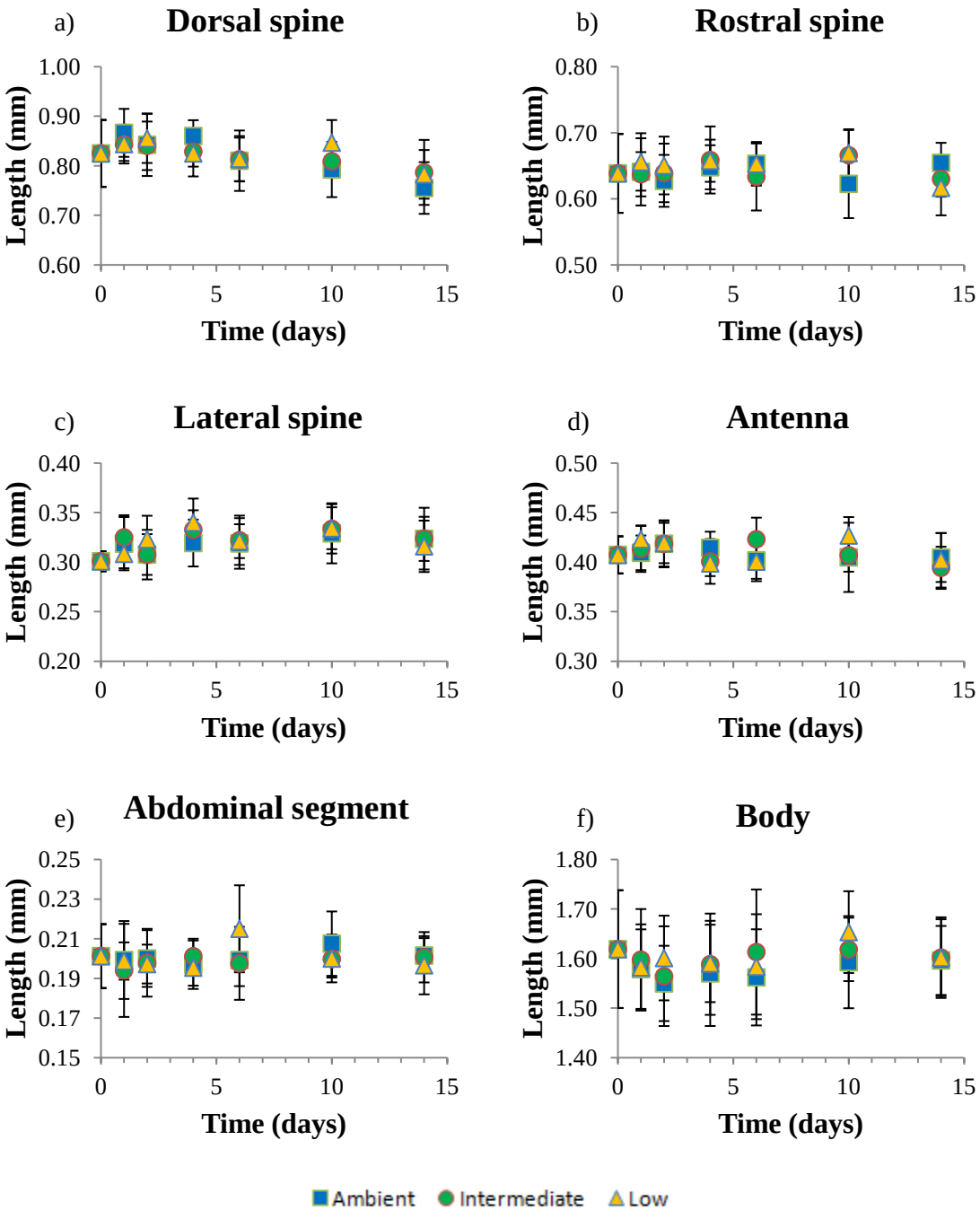

Figure A-5: Morphometric measurements of rock crab zoeae raised in jars. Measurements include (a) dorsal spine length, (b) rostral spine length, (c) lateral spine length, (d) antenna length, (e) length of the abdominal segment and (f) total body length. Error bars represent ± one standard deviation.

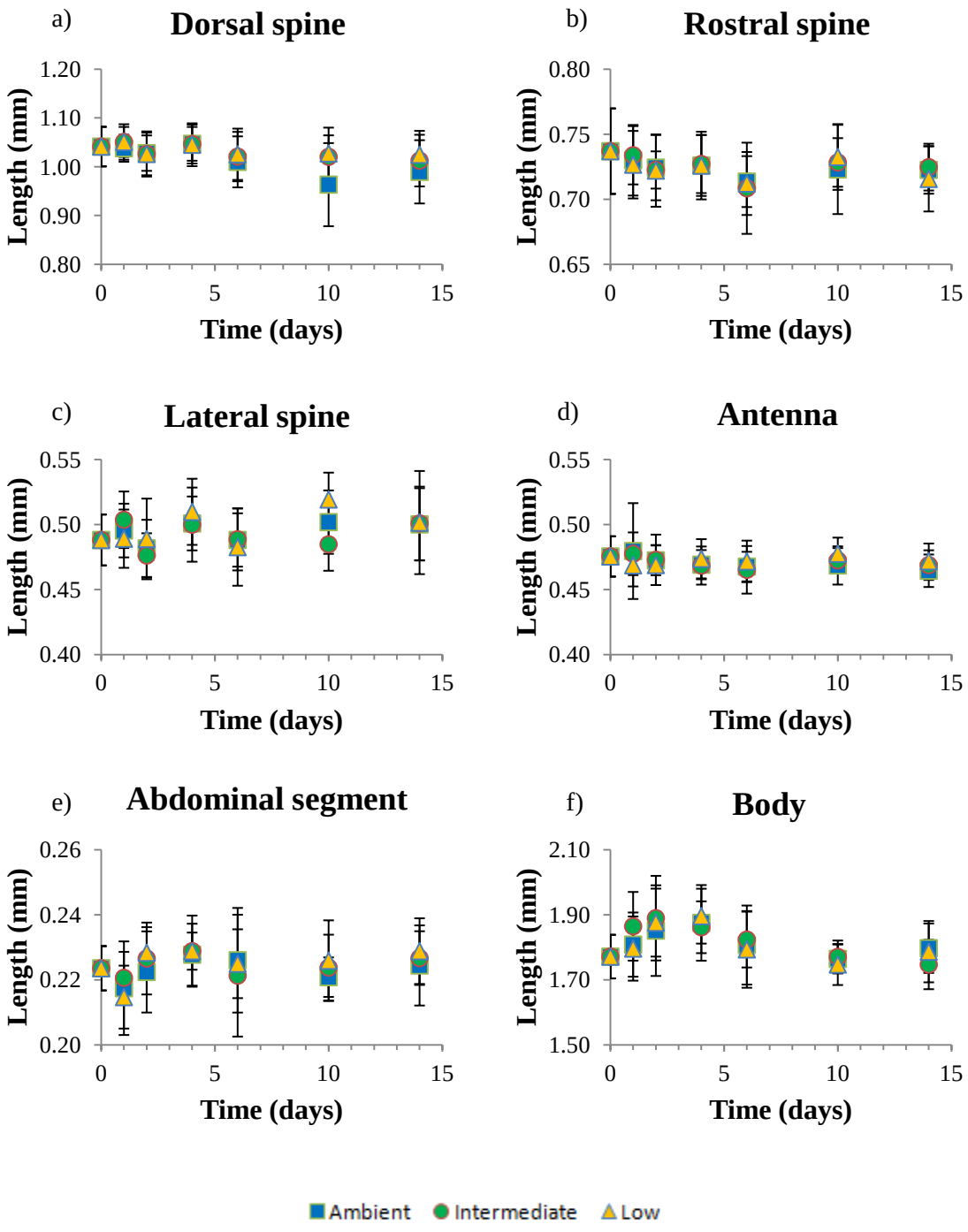

Figure A-6: Morphometric measurements of Dungeness crab zoeae raised in jars. Measurements include (a) dorsal spine length, (b) rostral spine length, (c) lateral spine length, (d) antenna length, (e) length of the abdominal segment and (f) total body length. Error bars represent ± one standard deviation.